Aptamer Biotechnology –

The Use of an Antibody Like Nucleic Acid

Against Cytochrome *c*

by

Lau Pui Man Irene, B. Sc. (Hons.),
The Chinese University of Hong Kong

A Thesis Submitted in Partial Fulfillment
of the Requirements for the Degree of
Master of Philosophy
in
Biochemistry

July, 2003

Acknowledgements

I would like to express my sincere gratitude to my supervisor, Prof. S. K. Kong for his invaluable ideas, guidance and support throughout my M. Phil. study.

I would also like to thank Dr. Y. K. Suen for his kind guidance and advice to my project.

Besides, I would like to give thanks to my dearest friends in Department of Biochemistry especially to Mandy Sung, Cindy Law, Raymond Chan, Patrick Chan, Wong Ho, Tsui Wai Lam, Ada Chui, Julia Lee, Macey Lee, Cindy Yuen, Ida Cheung, Rose Ong, Judy Wong and also friends in BMSB 514 for their support and spiritual encouragement during these two years of study.

Here, I also want to say thanks to Yan Yan and Ray Fung for their support started from my secondary school life.

Lastly, I would also like to thank my family for their continuous support in both my life and study.

Abbreviations

°C	Degree Celsius
%	Percentage
µg	Microgram
µl	Microliter
µm	Micron
µM	Micromolar
Apaf	Apoptosis Protease Activating Factor
ATP	Adenosine Triphosphate
bFGF	basic fibroblast growth factor
bp	Base Pairs
BSA	Bovine Serum Albumin
BrdUrd	5-bromo-2'-deoxyuridine
Ca^{2+}	Calcium Ions
Caspase	Cysteinyl Aspartic Acid-Protease
Cyto c	Cytochrome c
dH_2O	Distilled Water
DNA	Deoxyribonucleic Acid
dNTP	Deoxynucleic Triphosphate
DTT	Dithiothreitol
ECL	Enhanced Chemiluminescence
EDTA	Ethylenediaminetetraacetic Acid
ELISA	Enzyme Linked Immunosorbent Assay
EtBr	Ethidium Bromide
EtOH	Ethanol

FBS	Fetal Bovine Serum
FITC	Fluorescein Isothiocyanate
H_2O_2	Hydrogen Peroxide
HEPES	N-[2-Hydroxyethyl]piperazine-N'-[2-Ethanesulfonic Acid]
hr	Hour
HRP	Horseradish Peroxidase
kDa	Kilo Dalton
L	Liter
M	Molar
mg	Milligram
Mg^{2+}	Magnesium Ions
min	Minute
ml	Milliliter
mM	Millimolar
mV	Millivolt
no	Number
ng	Nanogram
nm	Nanometer
nM	Nanomolar
O.D.	Optical Density
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PMSF	Phenylmethyl-Sulfonyl Fluoride
PS	Phosphatidylserine
PVDF	Polyvinylidene Fluoride
RNA	Ribonucleic acid

RPMI	Roswell Park Memorial Institute
rpm	Revolution Per Minute
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction
S.D.	Standard Deviation
SDS	Sodium Dodecyl Sulfate
sec	Second
TBE	Tris Borate EDTA
TBS	Tris Buffered Saline
TBS-T	Tris Buffered Saline with Tween-20
TE	Tris-EDTA
TEMED	N, N, N', N'-Tetramethyl-Ethylene Diamine
TNF-α	Tumor Necrosis Factor-Alpha
UV	Ultraviolet
V	Volt
v/v	Volume by Volume
w/v	Weight by Volume

Abstract

Nucleic acids, deoxyribonucleic acid (DNA) and ribonucleic acid (RNA), are polymers of nucleotides. They store, transmit, and translate genetic information in living organisms. They are not linear molecules but fold into complex, sequence-dependent, three-dimensional shapes. These sequence-dependent shapes provide the molecules a capability to bind target molecules and make them like antibodies.

The oligonucleotides which have the ability to recognize and bind ligand molecules with a high affinity and specificity are termed 'Aptamer'. They can be RNA, single or double-stranded DNA. By SELEX (Systematic Evolution of Ligands by EX-potential enrichment), a process similar to the clonal selection and clonal expansion in our immune system, aptamers can be selected from a randomized oligonucleotide library containing about 10^{14} individual sequences and a large amount of variants can be obtained with subsequent rounds of amplification.

Up to now, aptamers have been designed and found to bind a number of targets such as dyes, drugs, nucleotides, proteins, intact cells etc.. With their high specificity and affinity, aptamers against disease-associated proteins can be used as therapeutic and diagnostic agents.

In my project, polyclonal and monoclonal aptamers in terms of nucleic acid

sequence were selected by using SELEX and their binding specificity was examined by Western blotting. The target molecule in our project was cytochrome *c* which is an important protein in apoptosis. Cytochrome *c* is a small, soluble protein with size of 15 kDa. It locates within the intermembrane space of the mitochondria. Upon apoptosis, cytochrome *c* is released from the intermembrane space of mitochondria to the cytosol and then activates the downstream caspase cascade to execute apoptosis.

In my study, 9 monoclonal aptamers were selected from a pool of oligonucleotides with 40-random-nucleotides sequence between the flanked 3' and 5' ends. These selected aptamers was found to bind to cytochrome *c* with non-specific bindings to other cellular proteins in the Western blot analysis. To eliminate the non-specific bindings, methods such as counter selection, primer testing and different blocking agents were tried. Eventually, one of the monoclonal aptamers cy-3 was tested for the semi-quantification of the release of cytochrome *c* from mitochondria in L929 cells after treatment with TNF-α. Results were compared with the conventional Western blot analysis by anti-cytochrome *c* antibody. Our results indicate that cy-3 could differentiate the intact cytochrome *c* and the one released from the mitochondria of apoptotic cells. These findings can help us to delineate the mechanism of cytochrome *c* release during apoptosis.

論文題目: 寡核苷酸配體生物技術-一個仿抗體的核酸分子之運用

學生姓名: 劉佩雯

導演姓名: 江紹佳教授

修業學位: 哲學碩士

學系: 生物化學

肄業日期: 二零零三年七月

摘要:

核酸 (nucleic acid) 包括去氧核糖核酸 (DNA) 及核糖核酸 (RNA)爲核苷酸之聚合物。於生物體中，它們負責儲存、傳遞及翻譯個體的基因訊息。一直以來，人們普遍地認爲核酸是線狀的。事實上，它們擁有複雜而且以其核苷酸序列作爲依據之立體結構。它們這種以核苷酸之序列作爲依據之結構爲它們帶來了與其靶分子結合之能力－如抗體與抗原結合之能力。

那些能與其靶分子以高親和力和高專一性結合的寡核苷酸被稱爲 '寡核苷酸配體'(aptamer)。它們可以是核糖核酸，也可以是單鏈或雙鏈的去氧核糖核酸。篩選合適之寡核苷酸配體的過程稱爲 SELEX (配體指數級富集系統進化)，而這一個過程與免疫系統中的克隆選擇 (clonal selection) 及克隆擴充 (clonal expansion) 很相似。經過 SELEX 一連串的選取及倍增，大量之寡核苷酸配體變體便可從一個擁有 10^{14} 個別不同核苷酸序列之隨機寡核苷酸庫中選取出來。

至今爲止，已有大量能與不同的靶分子結合的寡核苷酸配體被篩選出，這些靶分子包括:染料、藥物、核苷酸、蛋白質及完整的細胞等等。以寡核苷酸配體的高親和力和專一性，能和與病症有關的蛋白質結合的寡核苷酸配體可作爲治療或診斷的有效工具。

在此項研究中，多元性及單元性繁殖系的寡核苷酸配體在 SELEX 技術下從隨機寡核苷酸庫篩選出來並已用蛋白質印漬法 (Western blotting) 確認其靶分子。靶分子爲凋亡過程中一個標示性的蛋白質，細胞色素 *c* (cytochrome *c*)。細胞色素 *c* 是一個位於粒線體細胞內壁，分子量爲 15 kDa 的水溶性蛋白。在凋亡過程中，細胞色素 *c* 從粒線體細胞內壁中釋放到細胞溶質，隨後激活 caspase 反應並導致細胞凋亡。

在研究過程中，9 個單元性繁殖系的寡核苷酸配體已從一個寡核苷酸庫擁有由兩端的固定序列和中間 40 個隨機核苷酸序列組中篩選出來。由結果可見，篩選出來之寡核苷酸配體除了能與其靶分子細胞色素 *c* 結合外，亦與一些細胞溶質內的蛋白作非特定性的結合。我們嘗試用一些方法如相向篩選、引物測試及不同阻礙物等去除這一些非特定性的結合。最後，我們利用其中之一個單元性繁殖系的寡核苷酸配體 cy-3 對經過腫瘤壞死因子 (TNF-α) 處理過之 L929 細胞的粒線體中釋放的細胞色素 *c* 進行半定量分析，再將這些結果與抗細胞色素 *c* 抗體之結果作比較。我們的結果顯示了單元性繁殖系的寡核苷酸配體 cy-3 能區別完整的細胞色素 *c* 與從凋亡細胞粒線體釋出之細胞色素 *c*。

List of Figures

List of Tables

Contents

Chapter 1
Introduction

1.1 Introduction

The nucleic acids, ribonucleic acid (RNA) and deoxyribonucleic acid (DNA), are molecular substance to store genetic information. Genetic information flows from DNA to RNA by transcription and then to functional protein by translation. They had long been thought as solely an inert database for the genetic information with limited biological functions since they apparently lack of tertiary structural diversity.

1.1.1. Therapeutic uses of nucleic acids

Now, nucleic acids are being considered as therapeutic agents, either to interfere with the function of specific genes or to bind to specific proteins. Interference can occur either at mRNA level using antisense oligonucleotides (Hayward *et al.*, 2003) or RNA interference (RNAi) (Grzmil *et al.*, 2003); or at protein level by aptamers (Chen *et al.*, 2003).

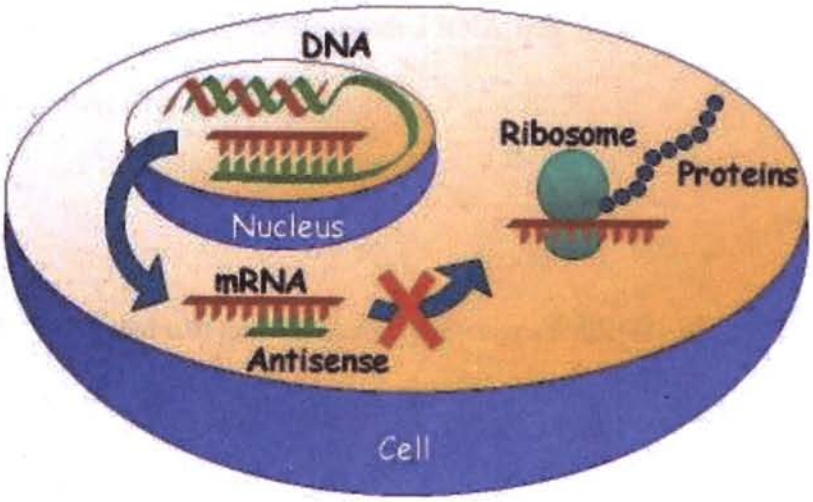
1.1.1.1. Antisense Oligonucleotides

After transcription, messenger RNA (mRNA) produced as a single-stranded molecule and its sequence of nucleotides is called 'sense' because it generates a gene product (protein). Normally, its nucleotides are 'read' by transfer RNA (tRNA) with anticodons as the ribosome walks along the mRNA during translation. However, RNA can form duplexes just as DNA does in the presence of a second stranded RNA whose sequence is complementary to the first strand. The second strand is called the antisense strand because its sequence of nucleotides is complementary to the original 'sense' one. When mRNA forms a duplex with a complementary antisense RNA sequence, translation is blocked. This occurs because the ribosome cannot read the

nucleotides in the mRNA. Alternatively, RNA in duplex form is rapidly degraded by ribonucleases in the cell.

With recombinant DNA technology, synthetic genes (DNA) encoding antisense RNA molecules can be introduced into organisms. For example, an artificial gene can be constructed to be carried in the genome (DNA) and is transcribed into an antisense RNA which is complementary to the mRNA of target. Antisense RNA can be used as a tool for therapy. And now, antisense RNA that is complementary to the proto-oncogene BCL-2 is being examined as a possible therapy for certain B-cell lymphomas and leukemias (Capaccioli *et al.*, 1996).

There is another similar strategy of interference called antisense oligodeoxynucleotides (ODNs). ODNs are synthetic DNA with 15-20 bases in length. Their sequence (3′ -> 5′) is antisense and is complementary to the sequence of a sense mRNA. Therefore, they can form duplex with mRNA and also block its translation. Antisense ODNs was tested against HIV-1 (Zamecnik *et al.*, 1986; Freund *et al.*, 2001), cytomegalovirus (CMV), asthma (Ball *et al.*, 2003) and certain cancers (Baron *et al.*, 2003; Leamon *et al.*, 2003) etc.. The U. S. FDA has approved fomivirsen, an antisense ODN that degrades the mRNA of two essential viral proteins for the treatment of CMV infections in the eye (Grillone *et al.*, 2001).



http://perso.club-internet.fr/ajetudes/nano/antisense.htm

Fig. 1.1. Antisense oligonucleotides

Antisense oligonucleotides are synthetic RNA or DNA fragments which are complementary to mRNAs. After the introduction into cells, they bind to their complementary mRNA and, block the corresponding protein translation.

1.1.1.2. RNA Interference

RNA molecules appeared in the cytoplasm normally are in single-stranded form. If a RNA molecule is in the form of duplex, an enzyme (the one in Drosophila has been named Dicer) usually cuts this double-stranded RNA (dsRNA) into fragments of 21-23 base pairs (~ 2 turns of a double helix) (Zamore *et al.*, 2000).

The two strands of each fragment then separate and the antisense strand can bind to the complementary sense sequence of a new mRNA molecule. This then elicits the cutting of the mRNA and destroys its ability to be translated into protein. Because of this activity, the double-stranded RNA fragment has been named "short (or small) interfering RNA" (siRNA).

A DNA vector that can continuously synthesizes a siRNA corresponding to the gene that it wants to block has been reported in mammalian cells (Brummelkamp *et. al.* 2002).

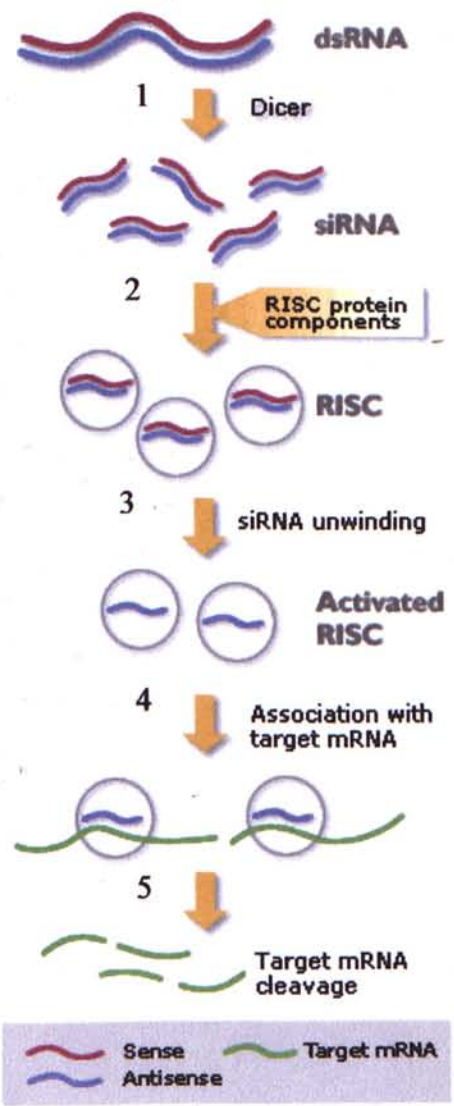


Fig. 1.2 Mechanism of RNAi

dsRNAs (typically >200 nucleotides) can be used to suppress the expression of target genes in many organisms and cell types. After introducing into cells, the dsRNAs enter the RNA interference (RNAi) pathway, a common cellular pathway. First, the dsRNAs were cut into 20-25 nucleotides small interfering RNAs (siRNAs) by an RNase III-like enzyme called Dicer (1). Then, the siRNAs form endoribonuclease-containing complexes known as RNA-induced silencing complexes (RISCs) (2). The siRNA strands are then unwound and became single stranded (3). The antisense siRNA strands subsequently guide the RISCs to complementary RNA molecules (4), where they cut the cognate RNA (5).

http://www.ambion.com/techlib/append/RNAi_mechanism.html

1.1.1.3. Aptamer

In the early of 1990s, natural occurring RNAs were found to bind small molecules. For example, the group I introns can bind guanosine nucleotides with a k_d of approximately 0.5 mM (Herschlag and Cech, 1990). Also, part of the binding site has been found to bind a base triple between a G-C base pair on the RNA.

Small nucleic acids were then found to fold into complicated 3-dimensional shapes which have binding specificities to many small molecules (Ellington *et al.*, 1992; Gold, 1995).

Oligonucleotides with selective binding capability which is selected by the combinatorial chemistry method SELEX (see later) from a pool of random nucleotide library is called 'Aptamer' or oligobody. Normally, they have a unique sequence that can fold into a specific 3-dimensional structure for ligand binding with a high affinity and specificity. The term 'Aptamer' is derived from a Latin word "aptus" which means "to fit" (Ellington, *et al.*, 1990).

1.2 Selection of aptamer

In 1990, RNAs with specific binding properties was firstly isolated from libraries of random RNAs by Tuerk and Gold (1990) and by Ellington and Szostak (1990). The technique of selection of these RNAs was called *in vitro* selection or SELEX (Systematic Evolution of Ligands by Exponential Enrichment) (Ellington and Szostak, 1990).

1.2.1. SELEX 'Systematic Evolution of Ligands by EXponential enrichment'

Using SELEX process, oligonucleotides can be selected efficiently from randomized libraries of RNA, DNA, or modified nucleic acids that bind target molecules with high affinity and specificity. This process started from a pool of oligonucleotides with random sequences in the core with two flanked known sequences and involves steps of selection and amplification.

The randomization of a sequence from 25 up to 100 nucleotides in length has been used to produce target-specific aptamers. Because the three-dimensional structure of short single-stranded nucleic acids are dictated by their sequences, such a library contains a large number of molecular shapes or conformations. For the diversity, the target recognition increases with the length of random sequence. By composed of the 4 different nucleotides, the diversity of the sequence in the library should be 4^n (where n is the number of nucleotide in the random sequence). For example, a library containing a 40-nucleotide random region is represented by 1.2×10^{24} individual sequences ($4^{40} = 1.2 \times 10^{24}$). However, in practice, due to the chemistry limitation, the complexity of oligonucleotides library is limited to 10^{14} to 10^{15} individual sequences.

1.2.1.1. *In vitro* selection

In vitro selection is a step to separate the oligonucleotides with high affinity to its ligand from the random library. This can be done by using affinity chromatography (Deng *et al.*, 2001), immuno-precipitation by magnetic beads (Bruno *et al.*, 2002), electrophoretic mobility shift assay, filter-binding techniques (Gal, 1997), or functional activity. For the selection using solid support for separation, target molecules are first conjugated on solid support like PVDF membrane, agarose beads or magnetic beads (Ishizaki *et al.*, 1996).

Selection can be initiated by allowing the oligonucleotide library to react with the target molecules under a favorite binding condition (suitable buffer, pH and temperature). The aptamers selected under the selection condition must be used in such condition afterwards. (Lorsch *et al.*, 1994)

During the selection, the unbound oligonucleotides are separated from those bound to the target molecules by washing the solid support with binding buffer; by filtration with the use of suitable filter or by the different mobility between the complexes and the free unit in a separating gel. Subsequently, the bound oligonucleotides are then eluted and are eventually purified from the target molecules and precipitated by ethanol.

1.2.1.2. Amplification

The bound oligonucleotides isolated are then amplified by reverse transcription and PCR (for RNA-based libraries) or just asymmetric PCR (for DNA-based libraries) (Innis *et al.*, 1988) with primer pairs which are complementary to the

flanked ends of the oligonucleotides pool. Then a pool enriched in sequences that bind to the target protein is generated. This library is then transcribed *in vitro* (for RNA-based libraries), or its strands are separated (for DNA libraries) to generate molecules for use in the next round of selection.

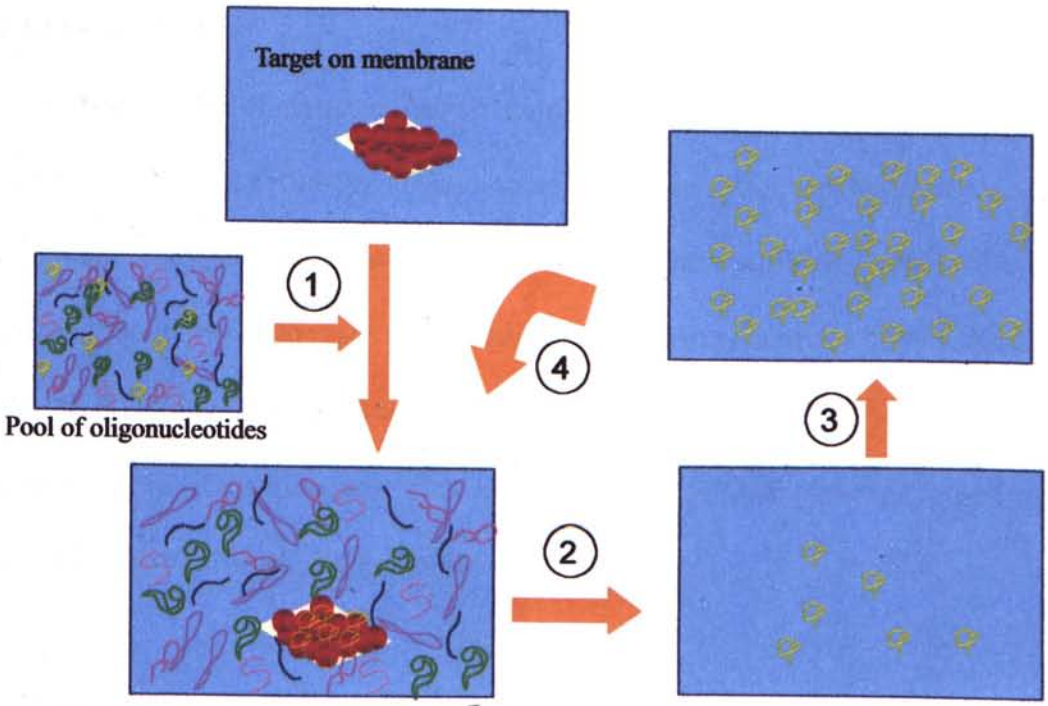


Fig. 1.3. Aptamer selection scheme showing one round of an *in vitro* selection against a protein target dotted on PVDF membrane.
(1) A library of oligonucleotides first incubates with targets dotted on a membrane. (2) After washing, the bound oligonucleotides are eluted from the targets. (3) Asymmetry PCR is performed to amplify the selected oligonucleotides. One round of selection is completed. (4) Another round of selection then starts with the amplified oligonucleotides. Several rounds of selection and amplification are carried out.

1.2.1.3. Monoclonal aptamer

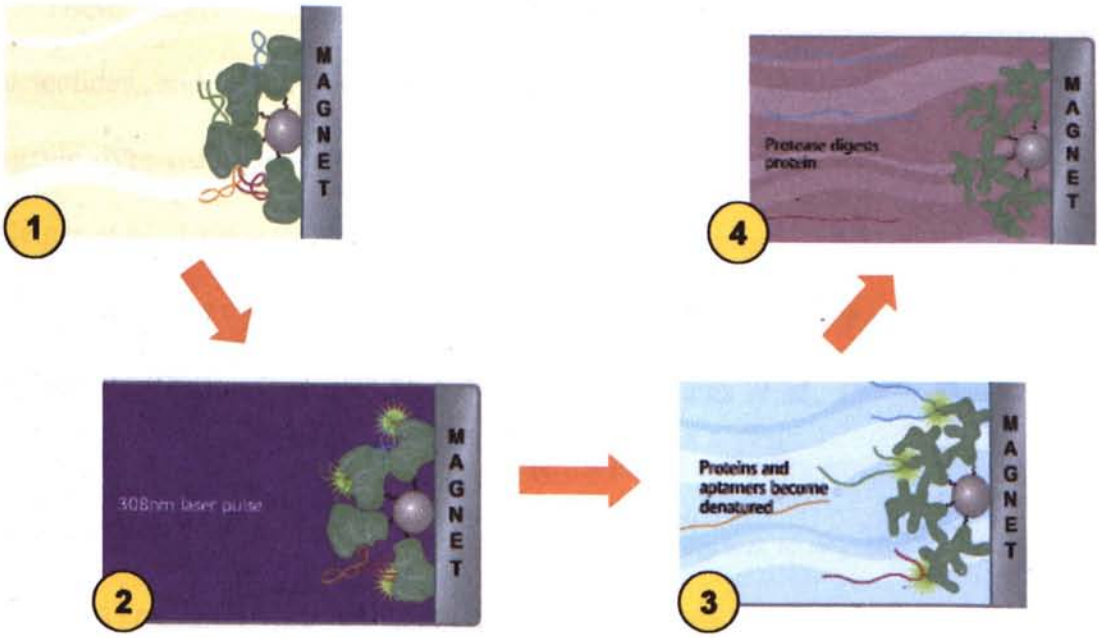
After several rounds of selection and amplification, usually 5-15 rounds, SELEX is completed and polyclonal nucleic acids with high affinity and specificity are selected against the target. The pool of the nucleic acids isolated can then be cloned and monoclonal nucleic acid can be obtained.

1.2.2. Photo-SELEX

A special type of aptamer known as photo-aptamer, is developed that bears photocross-linking functionality. Photo-aptamer can be generated by incorporating the 5-halo-deoxyuridine such as 5-bromo-2'-deoxyuridine (BrdUrd) or 5-iodo-2'-deoxyuridine in place of thymidine in the oligonucleotides library. And the photo-cross-linking usually takes place between the C-5 position of the nucleobase and an electron-rich amino acid such as Tyr and Trp at close proximity under UV illumination (Smith *et al.*, 2003; Golden *et al.*, 2000).

During selection, the pool of photo-aptamers is first incubated with the target. After binding, the solution was irradiated by a short wave (254 or 266 nm) UV light or 308nm light for cross-linking. UV light or light at 308 nm elicits photoelectron transfer from a nearby electron donor to the bromouracil base via either excitation of the BrdUrd, excitation of the electron donor, or excitation of a BrdUrd-electron donor charge transfer state and finally, unbreakable cross-linking forms. Amino acids residues that can serve as electron donors in BrdUrd photocross-linking include Tyr, Trp, His, Phe, Cys, Cys-Cys, and Met. Among these residues, Tyr and Trp are excited at 308 nm.

Photocross-linking can enhance the specificity of the aptamer-protein interaction. Although a protein may bind an aptamer nonspecifically, the probability that an appropriate amino acid would be positioned to cross-link with a BrdUrd residue would be low.



Modified from http://www.somalogic.com/science/photoselex.html

Fig. 1.4. Photo-SELEX scheme

(1) The oligonucleotides first incubate and bind to the target. (2) After washing, laser pulse or UV light is applied. Photo-cross linkages only form between those oligonucleotides bind to their targets in the right position. (3) At high temperature, proteins and aptamers become denatured and those without cross linkage were washed away. (4) The oligonucleotides isolated are then released by protease which digests the target molecules.

1.3 Examples of target molecules of aptamers

Up to now, aptamers have been developed for a wide variety of targets with dissociate constant typically range from nanomolar to picomolar or even sub picromolar affinity (Burgstaller *et al.*, 2002; Pavski *et al.*, 2003; Uphoff *et al.*, 1996).

These target molecules include proteins, peptides, dyes, amino acids, nucleotides, and drugs (Deng *et al.*, 2001), metal ions (Hofmann *et al.*, 1997), organic dyes (Ellington *et al.*, 1992), drugs (Mannironi *et al.*, 1997), amino acids (Geiger *et al.*, 1996), co-factors (Wilson *et al.*, 1998), aminoglycosides (Wang *et al.*, 1996), antibiotics (Wallace *et al.*, 1998), nucleotide base analogs (Kiga *et al.*, 1998), nucleotides (Haller *et al.*, 1997) and peptides (Williams *et al.*, 1997). There are a growing number of protein targets to which selected aptamers bind. These include enzymes (Bridonneau *et al.*, 1998), growth factors (Pegratis *et al.*, 1997), antibodies (Wiegand *et al.*, 1996), gene regulatory factors (Lochrie *et al.*, 1997), cell adhesion molecules (Kraus *et al.*, 1998), and lectins (Bridonneau *et al.*, 1999). Some of the examples are summarized in Table 1.1.

Table 1.1 Examples of aptamers selected

Type	Example	Reference
Enzyme	Non-structural protein 3 protease of hepatitis C virus	Nishikawa *et al.*, 2003
	ERK2	Bianchini *et al.*, 2001
Growth factor	Basic fibroblast growth Factor (bFGF)	Golden *et al.*, 2000
	Keratinocyte growth factor (KGF)	Pagratis *et al.*, 1997
Peptide	Coagulation factor IXa	Rusconi *et al.*, 2002
	Nucleocapsid protein of HIV-1	Kim *et al.*, 2002
Antibodies	Myasthenic Autoantibodies	Hwang *et al.*, 2002
Antibiotic	Tetracycline	Berens *et al.*, 2001
	Streptomycin	Wallace *et al.*, 1998
Dyes	Sulforhodamine B	Wilson *et al.*, 1998
	Cibacron Blue 3GA, Reactive Red 120, Reactive Green 19 etc.	Elington *et al.*, 1990
Amino acids	Arginine	Nolte *et al.*, 1996
	Isoleucine	Majerfeld *et al.*, 1998
Nucleotides	AMP	Burke *et al.*, 1997

1.4 Applications of aptamer

Until now, many aptamers were selected against different targets including disease-related proteins and analytical targets. Many different applications were also developed. These applications of aptamers include rapid target validation (Rhodes *et al.*, 2000), pathway dissection (Seiwert *et al.*, 2000), the discovery of diagnostic reagents (Hicke *et al.*, 2000; Brody *et al.*, 1999) and novel therapeutics (Floege *et al.*, 1999).

1.4.1. Detection of aptamer

There are three methods commonly used for the detection of aptamers, They are radiolabeling, biotin – streptavidin system and fluorescent labeling.

For radiolabeling, radiolabeled ATP is used and incorporated into aptamer during amplification by PCR. With radioactive aptamers, the amount of bound aptamers with the target obtained by filtration can be easily estimated by scintillation counting. Alternatively, the presence of aptamer after probing protein on membranes or mobility shift in gel can be easily detected by exposing the complex to x-ray films.

For the biotin – streptavidin detection system (Gretch *et al.*, 1987), aptamers are first amplified by PCR with the use of biotinylated 5' forward primer. After several rounds of amplification, the PCR products are all biotinylated. After the binding of aptamer to the protein, the biotinylated aptamer can be detected by streptavidin. The streptavidin usually conjugates with an enzyme such as horseradish peroxidase or alkaline phosphatase. In the presence of suitable substrate, chemiluminescence signal

or colour change can be measured by X-ray films or colorimetric reader.

The use of fluorescent labeling is similar to that of biotin. In the PCR, FITC conjugated 5' forward primers are used. The FITC conjugated PCR products can be used for target labeling. Signals on the aptamers can then be detected directly by x-ray films or fluorometric reader. However, photo-bleaching may occur during the processing of the fluoro-aptamer.

1.4.2. Examples of diagnostic use

With their easy labeling and their high specificity and high affinity, DNA aptamers been exploited in diagnostic applications with biosensor (Potyrailo *et al.*, 1998) and flow cytometer (Davis *et al.*, 1996).

1.4.2.1. Aptamer against theophylline with high specificity

One of the important features of antibody or aptamer is the binding specificity to target molecules or antigens. Theoretically, the diversity of antigen-binding sites of aptamers is equivalent to, if not greater than that of antibody. Below are some of the examples showing aptamer specificity.

Theophylline is a natural occurring alkaloid that is used widely as bronchidilator in the treatment of asthma, bronchitis, and emphysema. However, its therapeutic index is narrow and the serum level should be monitored carefully to avoid toxicity (Hendeles *et al.*, 1983). In serum, the presence of theobromine (3, 7-dimethylxanthine), which is chemically similar to theophylline may affect the result of monitoring theophylline serum level, like caffeine. Diagnostic methods

must be developed to discriminate efficiently among these compounds.

Species of RNA that bind with high affinity and specificity to theophylline were identified by SELEX from a pool of 10^{14} oligonucleotide library with 40 random nucleotides (Jenison *et al.*, 1994). One of the RNA aptamers selected bound to theophylline with a dissociation constant K_d of 0.1 μM. When compared the binding of this RNA aptamer to caffeine which differs from theophylline only by a methyl group at nitrogen atom N-7, the binding affinity with theophylline is 10,000-fold greater than that of caffeine suggesting that the binding specificity of aptamer is very high.

The discrimination of the aptamers between theophylline and caffeine can be achieved by "counter-SELEX". In the selection of aptamer against theophylline, counter selection was done after the fifth round of selection by eluting those bound aptamers by caffeine before using theophylline. The aptamers eluted by caffeine were then discarded and those eluted by theophylline were collected. The aptamers so selected can discriminate theophylline from caffeine.

1.4.2.2. Aptamer chip

Recently, aptamers for different targets are synthesized and dotted on a micro-chip or an array. By using arrays, it ultimately allows the simultaneous measurement of the concentrations of hundreds or thousands of targets in a broad range of sample types. And thus they can be used for diagnosis of disease. Now, there are three different types of array, DNA array, protein array and aptamer array.

For the DNA array, genes are dotted on the specific site of a chip. After exposure to an mRNA sample, the complementary strands are hybridized with DNA. With appropriate signal generation system, signals such as fluorescence can be generated from the chip and the change of mRNA expression level can be determined.

For protein and aptamer array, antibodies or aptamers for specific targets are dotted on the chip. After exposure to the samples such as cell lysate, blood or urine, the specific targets are bound on the chip. For protein chip, the detection of the specific target should be done by the aid of secondary antibody conjugated with enzyme or fluorescent indicator. While for the aptamer chip, since the aptamer are not protein in nature, the presence of the protein bound on the chip can easily be detected by common protein stains. With a database generated from the pattern of normal and disease samples, the disease state of a patient can be easily analysized by comparing the patients' pattern with the database (Kodadek, 2002; Petach *et al.*, 2002; Walter *et al.*, 2002).

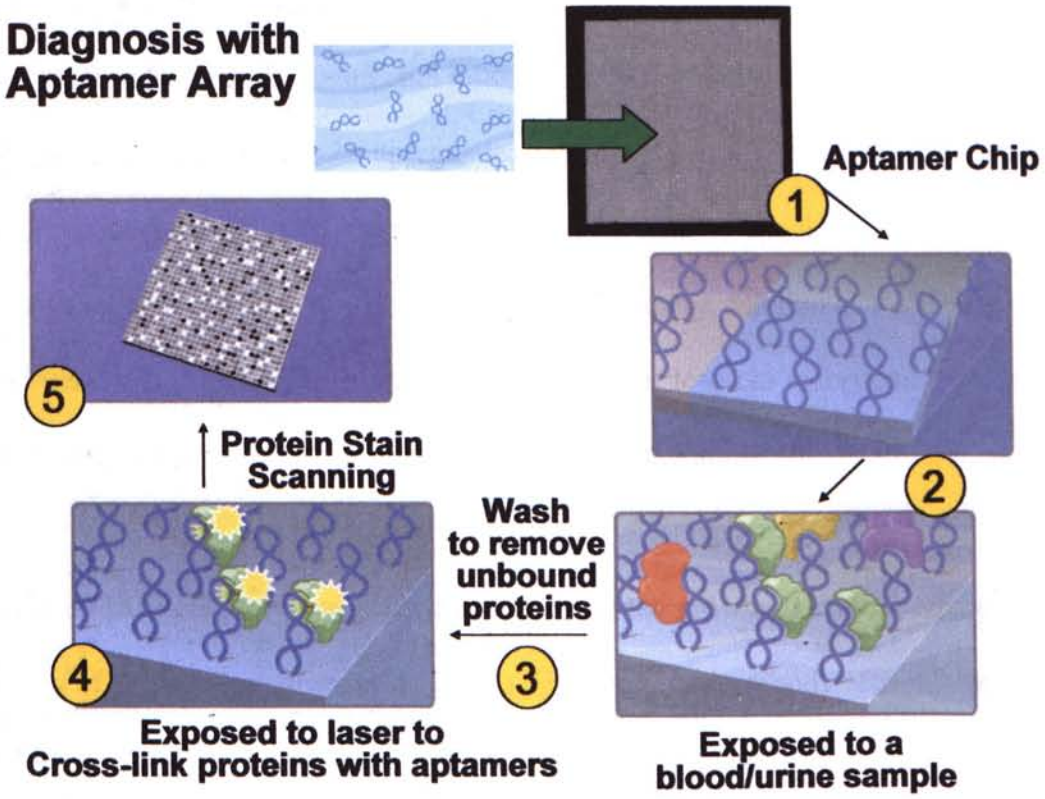


Fig. 1.5. Diagnostic use of aptamer – Aptamer array

For the diagnosis with aptamer array, aptamers are first dotted on a chip (1). Then the chip is exposed to a clinical sample such as urine or blood (2). After binding, the unbound proteins are washed away (3) and cross linking can be obtained by laser or UV exposure (4). The proteins on aptamers can then be stained (5). By this method, concentration of ten of thousands proteins can be measured simultaneously.

1.4.3. Examples of therapeutic use

1.4.3.1. Vascular endothelial growth factor (VEGF)

Aptamer can be used as a therapeutic agent. One of the advantages of using aptamers is their non-immunogenic feature due to their small size and similarity to endogenous molecules.

Aptamers against vascular endothelial growth factor (VEGF) is a good example. VEGF is an angiogenic promoter present in a wide variety of human tumors and is a well characterized specific endothelial mitogen and permeability factor (Leung *et al.*, 1989). Its level was found to be upregulated in the majority of human tumors. Also, it has been established recently that VEGF is a survival factor for endothelium in new blood vessels (Gerber *et al.*, 1998). It was found that a deletion of 1 allele in the VEGF gene with result in abnormal development that leads to embryonic lethality. Thus, VEGF is a potential target for novel anti-tumor therapies.

Anti-VEGF antibody was reported to suppress the growth of tumors effectively (Rowe *et al.*, 2000, Borgstrom *et al.*, 1998). However, this antibody recognizes all 4 isoforms of VEGF which are expressed by alternative splicing: VEGF 121, 165, 189 and 206 (Backer *et al.*, 2001). One of these, VEGF 165, appears to be expressed predominately in adult human tumors but its role in pediatric cancers has not been established. If other isoforms support the physiologic angiogenesis associated with tumour growth, narrowing the target to a tumor-associated isoform might provide benefits in pediatric patients.

Recently, an isoform-specific anti-VEGF 165 aptamer with high affinity and nuclease resistant property (fluoropyrimidine modified RNA-based aptamer) was characterized (Ruckman *et al.*, 1998). This specific aptamer is known as NX1838 and was found to suppress the primary tumor growth and metastasis of Wilms tumor through the inhibition of vascular cell proliferation and vascular permeability (Huang *et al.*, 2001, Ruckman *et al.*, 1998, Ostendorf *et al.*, 1999).

1.4.3.2. Aptamer as a reversible antagonists of coagulation factor IXa is another example to show the potential use of aptamers as therapeutic agents

The blockage of blood vessels by abnormal blood clots is one of the biggest causes of serious illness and premature death in Western societies. Normally, the patient is treated with heparin which is an effective anti-coagulant. However, heparin may induce an autoimmune disease called thrombocytopenia that resulted in a reduction in platelet count and prolonged subcutaneous bleeding. Also, heparin does not have rapidly acting antidote. It is therefore very dangerous as significant bleeding due to imbalance of coagulation and anti-coagulation may lead to an increase in morbidity and mortality of patient.

Aptamer specific for coagulation factor IXa which is the key factor of both the intrinsic and extrinsic pathway of the coagulation was developed (Rusconi *et al.*, 2002). The aptamer was selected from a 10^{14} combinatorial library of oligonucleotides with 40 random nucleotides sequence. After eight rounds of selection, aptamer that is 5,000 times more specific for Factor IXa than that for the structurally related factors such as Factor VIIa, Xa, XIa was selected. After the aptamer binds to the coagulant factor IXa, it blocks the activation of Factor IXa by Factor VIIa or prevents the activation of Factor X by Factor IXa together with Factor VIIIa.

The antidote which can reverse the anti-coagulant effect of the selected aptamer is also characterized. For an antidote to neutralize the effects of aptamers, it is only a sequence that is complementary to the critical and functional base pair of the aptamer. After the antidote binds with the aptamer, it blocks the binding site of the aptamer to Factor IXa, thus eliminating the anti-coagulant effect.

1.4.4. Problem faced by aptamer

1.4.4.1. Stability

For the use of aptamers with biological samples, nuclease attack is one of the major problems. Unmodified oligonucleotides, especially RNA, are degraded easily by nuclease which is commonly present in biological fluids. The *in vitro* half-life of a typical RNA oligonucleotide in plasma is a few seconds. This limits RNA aptamer to be used as measure analytes or therapeutics agents in biological fluids that contain nucleases.

However, this can be overcome by chemical modification of oligonucleotides. Since ribonucleases utilize the 2'-OH group for the cleavage of the adjacent phosphodiester bond and the nucleases that are most abundant in biological fluids appear to be pyrmidine-specific endonucleases, substitution of the 2'-position of pyrimidine nucleotides with 2'-amino (2'-NH_2) (Rusconi *et al.*, 2000), 2'-fluoro (2'-F), or a variety of 2'-O-alkyl moieties makes the oligonucleotides resistant to ribonucleases with an *in vitro* half-life in the 5-15 hour range (Jayasena *et al.*, 1999).

Synthetic phosphodiester-modified oligonucleotides such as phosphorothioate oligonucleotide (S-ODN) and phosphorodithioate oligonucleotide (S_2-ODN) analog can also increase nuclease resistance and can bind to proteins with enhanced affinity. Unfortunately, ODNs possessing high fractions of phosphorothioate or phosphorodithioate linkages appear to lose some of their specificity and are 'sticker' toward proteins (Yang *et al.*, 2002). Thus, the ratio of modification must be adjusted.

ODN modification makes aptamer suitable for both diagnostic and therapeutic

applications. When comparing unmodified RNA with unmodified DNA, unmodified DNA sequences are generally more nuclease resistant and the *in vitro* half-life of a typical DNA oligonucleotide in plasma is about 30 to 60 minutes. Therefore, it is reasonable to assume that DNA aptamers without further modifications can be used in diagnostic assays in which aptamers may come into contact with biological fluids for a brief period of time. If necessary, additional protection from exonucleases can be provided through terminal capping with small molecules such as an amino linker, a phosphate group, or an inverted thymidine residue. For most diagnostic formats, terminal modification of aptamers is a common practice and provides a route to conjugate aptamers either to reporter molecules or to solid supports.

Apart from modification, another possible way to protect RNA from nuclease degradation is by mirror image. This method is indirect and consists of two steps: in the first step, an aptamer is selected to bind the enantiomer of the target, then in a second step, the enantiomer of the aptamer is synthesized (from L-phosphoramidites) as a nuclease-insensitive ligand of the natural target. This mirror-image approach has been applied to L-arginine, D-adenosine, and the peptide hormone vasopressin (Famulok *et al.*, 2000).

1.4.4.2. Clearance from blood

Apart from stability, clearance from blood is another problem for the therapeutic use of aptamer. Due to the small size of an aptamer (an aptamer with 40 nucleotides in length is nearly 13200 Da in molecular weight), they are cleared from animals via the kidneys in minutes. Therefore, for a stable aptamer, the *in vivo* plasma half-life is governed by the rate of clearance from the animal.

The clearance rate of an aptamer can be altered by increasing its effective molecular size rationally, such as by the site-specific addition of various molecular weight polyethylene glycol (PEG) moieties or other hydrophobic groups, or by attachment of the aptamer to the surface of a liposome. After the modification, a given aptamer can be formulated to have a longer half-life.

1.5 Comparison between aptamer and antibody

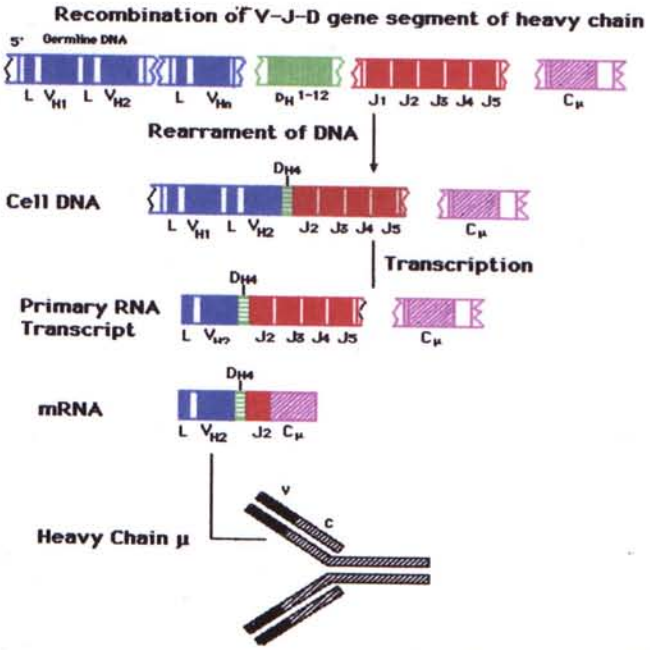
1.5.1. General comparison between aptamer and antibody

1.5.1.1. Diversity

Between aptamer and antibody, the most obvious difference is that aptamer is nucleic acid in nature while antibody is protein. This makes aptamer become more stable in extreme condition when compare with antibody. Also, direct protein staining can be applied in the diagnostic use of aptamer since it is not protein in nature. However, antibody can only be recognized by secondary antibody which conjugated with an enzyme and finally produces colour product or chemiluminescence. Moreover, the production of aptamers can be controlled more easily since they are selected *in vitro* while antibody is usually produced *in vivo*.

Antibody is composed of 2 heavy and 2 light chains. And their diversities depend on the combination of the gene segments of V, D, J and N region. Light chains consist of V and J gene segments at the variable region while heavy chains consist of V, D and J gene segments. By rearranging different segments of V, D, J and N regions in the pool of stem cells, a large diversity of light chains and heavy chains is resulted. With different combinations of light chain and heavy chain, diversity of about 1.4×10^{10} different antibodies is resulted (Fig. 1.6).

Aptamer is a single chain consists of a random region in two flanked ends. The diversity depends on the different arrangements of the four nucleotides in the random region. For a pool of oligonucleotides with 40 random nucleotides sequences, its sequence diversity is $4^{40} \approx 1.2 \times 10^{24}$. However, the diversity of the random sequences is limited to the DNA synthesis chemistry that is about 10^{15}.



http://ntri.tamuk.edu/immunology/abproduction2.html

Antibodies (BCRs)	Gene Segments	Combinations
V	32	
J	5	
N regions	100	1.6×10^4 L chains
V_H	50	
D_H	29	
J_H	6	
N regions	100	8.7×10^5 H chains
Any H chain with any kappa L chain		1.4×10^{10}

Fig. 1.6. The production of a heavy chain of IgG by rearrangement of V, D, J and N segments and the table below shows the diversity of antibody.

1.5.2. Specificity

From the pervious study, aptamers were shown to have high specificity. For the aptamer against VEGF therapeutic use, it can recognize the VEGF isoform 165 among 4 subtypes which are slightly different while antibody cannot differentiate the four isoforms.

1.5.3. Disadvantages of antibody

Antibodies are usually raised *in vivo* and their performances are dependent on the responsiveness of hosts. Poorly immunogenic and toxic molecules are difficult or impossible to raise antibody. Also, the use of antibodies is restricted by the *in vivo* parameters that antibodies are not compatible to extreme conditions other than physiological environment. Even the same type of antibody, different batches of antibody varies and immunoassays are required to be optimized with each new batch of reagents.

Production of monoclonal antibodies can be done *in vitro* with *in vitro* immunization. However, monoclonal antibody production is laborious and expensive. After screening a large number of colonies, target clone has to be expanded *in vivo* or *in vitro* for antibody production. From time to time, frozen stocks of antibody-producing cells must be stored at multiple sites in case of accidental losses or the death of cells lines. Still, hybridomas and animals must be kept alive during the production process.

Also, antibodies are large in size and complexity. This makes them difficult to be modified with enzymes or labeling agents. Sometimes, modification may reduce

the affinity of antibody. Also, antibodies tend to be denatured, they lose their structure when they are heated or exposed to extreme pH (Drolet *et al.*, 1996).

1.5.4. Advantages of aptamer

On the contrary, aptamers are selected *in vitro*, the processes do not involve animals, cells, or even *in vivo* condition. As a result, variation between individual animals or cells can be eliminated. Also, their productions are by chemical synthesis with extreme accuracy and reproducibility. Therefore, the product performance is expected to have no or little batch-to-batch variation. By *in vitro* selection, aptamers for molecules that are toxic or that do not elicit good immune responses can also be generated. By using *in vitro* selection, the properties of aptamers can be changed on demand according to the uses of aptamers. For example, aptamers can be selected to bind to a target in a nonphysiological buffer and at nonphysiological temperature.

After selection, once the sequences of aptamer have been identified, they can be synthesized in large quantities and are reproducible in any laboratory with stable performance. Furthermore, the price for aptamer production is relatively low.

Aptamers are usually 15 times smaller than that of a typical IgG antibody and they can be easily modified. For example, reporter molecules such as biotin and fluorescein can be conjugated to aptamers at precise locations. Different functional groups that allow subsequent modification of aptamers with other molecules can also be conjugated during the chemical synthesis of aptamers. Although modification of aptamer may also reduce the affinity, this can be overcome by 'Post-SELEX'. That means the high affinity aptamer with suitable modification can be selected again

with a modified polynucleotide library while the modification can be designed according to the final use of the aptamer.

Moreover, aptamers can undergo reversible denaturation that they can be renatured after exposure to denaturing conditions such as heat, salt concentration, pH of the medium, and chelating agents. Also, they are stable to long-term storage and can be transported at ambient temperature.

1.6 Project objectives

In this project, cytochrome *c* is the target of the aptamer selection.

Cytochrome *c* is a small, soluble protein of 15 kDa in size and is localized in the mitochondrial intermembrane space and is loosely attached to the surface of the inner membrane. On cytoplasmic ribosomes, apocytochrome *c* is synthesized and then translocated into the mitochondria. There is a covalent attachment of the apocytochrome *c* and the heme group that is synthesized in mitochondria and this creates functional cytochrome *c* (Vander Heiden and Thompson, 1999). Cytochrome *c* is a requisite component of the mitochondrial respiratory chain and participates in the electron transfer between complex III and complex IV of the respiratory chain in mitochondria for the production of ATP.

On the other hand, cytochrome *c* is released from the intermembrane space of mitochondria into the cytosol due to the opening of the mitochondrial permeability transition pores in response to a variety of apoptotic stimuli (Mignotte and Vayssiere, 1998; Single *et al.*, 1998; Skulachev, 1998). Cytosolic cytochrome *c* is an essential component of the vertebrate apoptosome which is composed of cytochrome *c*, Apaf-1, and procaspase-9 (Apaf-3). Interaction of these proteins leads to the activation of caspase-9, which in turn activates other caspases (such as caspase-3) ultimately resulting in cell death (Green and Reed, 1998). Therefore, the release of cytochrome *c* is one of the hallmarks of apoptosis.

For the time being, release of cytochrome *c* from apoptotic cells is detected by Western blot analysis with antibody. In this project, aptamers against cytochrome *c* were selected from a pool of oligonucleotides as a substitute of anti-cytochrome *c*

antibody. Also, we tried to streamline the process for selecting useful aptamer.

Chapter 2
Materials and Methods

2.1. Materials

2.1.1. Chemicals

Table 2.1
A list of the common name, chemical name and formula of chemicals used in this project.

Common name, Chemical name and formula	Formula Weight	Source
2-mercaptoethanol (C_2H_6OS)	78.13	Sigma Chemical Co., St. Louis, MO, USA
Acetate		
Acetic acid (CH_3COOH)	60.05	BDH Chemicals Ltd., Poole, England
Acrylamide/bis-acrylamide (30% solution, mix ratio 37.5:1)	/	Sigma Chemical Co., St. Louis, MO, USA
Agarose	/	Sigma Chemical Co., St. Louis, MO, USA
Albumin, Bovine (BSA, initial fractionation by cold alcohol precipitation, Fraction V 96-99% albumin)	/	Sigma Chemical Co., St. Louis, MO, USA
Ammonium persulfate	228.2	BIO-RAD Laboratories
Anti-cytochrome *c* antibody Clone no. 7H8.2C12	/	Pharmingen
Streptavidin antibody-HRP conjugated antibody (mouse horseradish peroxidase conjugated)	/	Pharmingen
Anti-mouse-HRP conjugated antibody (mouse horseradish peroxidase conjugated)	/	BIO-RAD Laboratories
Aprotinin (3 – 7 TIU (trypsin inhibitor unit))	/	Sigma Chemical Co., St. Louis, MO, USA
BCA (Bicinchoninic acid solution)	/	Sigma Chemical Co., St. Louis, MO, USA
Bromophenol blue ($C_{19}H_9Br_4O_5SNa$)	691.9	Sigma Chemical Co., St. Louis, MO, USA
Chloroform ($CHCl_3$)	119.38	Merck, Germany

Commassie brilliant blue R-250 ($C_{45}H_{44}N_5O_7S_2Na$)	826.0	USB (United States Biochemical), Cleveland, OH, USA
Cytochrome *c*, Horse	12,384	Sigma Chemical Co., St. Louis, MO, USA
Deoxynucleotide set (dNTP) (dATP, dTTP, dGTP, dCTP)	/	Sangon Biologic Engineering Technology and Service Co. Ltd., .Shanghai, China
Digitonin ($C_{56}H_{92}O_{29}$)	1,228	Merck, Germany
DNA marker (25 base pair)	/	GibcoBRL, Life Technologies Inc.
ECL reagents (Enhanced chemiluminescence reagents)	/	Amersham Pharmacia Biotech
Ethanol (EtOH) (C_2H_5OH)	46.07	BDH Chemicals Ltd., Poole, England
Ethanolamine		
Ethidium bromide (EtBr)	394	Molecular Probes, Eugene, Oregon, USA
Ethylenediaminetetraacetic acid (EDTA) ($C_{10}H_{16}N_2O_8$)	292.2	Sigma Chemical Co., St. Louis, MO, USA
Ethyleneglycol-*bis*(b-aminoethyl)-N,N,N′,N′-tetraacetic Acid (EGTA) ($C_{14}H_{24}N_2O_{10}$)	380	Amersham Pharmacia Biotech
Fetal Bovine Serum (FBS)	/	GibcoBRL, Life Technologies Inc.
Ficoll	400,000	Amersham Pharmacia Biotech
Formamide (CH_3NO)	45	Sigma Chemical Co., St. Louis, MO, USA
Glycine ($H_2NCH_2CO_2H$)	75.07	USB (United States Biochemical), Cleveland, OH, USA
HEPES (N-[2-hydroxyethl]piperazine-N'-[2-ethanesulfonic acid])	238.8	Sigma Chemical Co., St. Louis, MO, USA
Hydrochloric acid (HCl)	36.45	BDH Chemicals Ltd., Poole, England
Isoamyl alcohol (3-methyl butanol-(1)) ($C_5H_{12}O$)	88.15	Riedel-de Haen, AG, Germany
Isopropanol ($(CH_3)_2CHOH$)	60.1	BDH Chemicals Ltd., Poole, England
Isopropyl-b-D-thiogalactopyranoside (IPTG)	282.4	USB (United States Biochemical), Cleveland, OH, USA

Leupeptin ($C_{20}H_{38}N_6O_4$)	463	Sigma Chemical Co., St. Louis, MO, USA
Lysine ((L-2,6-Diaminohexanoic acid) monohydrochloric)	182.6	Sigma Chemical Co., St. Louis, MO, USA
Low range marker for Western blot	/	BIO-RAD Laboratories
Magnesium chloride ($MgCl_2$)	203.3	Sigma Chemical Co., St. Louis, MO, USA
Magnesium sulfate-7-hydrate ($MgSO_4$-$7H_2O$)	246.48	BDH Chemicals Ltd., Poole, England
Methanol (CH_3OH)	32.04	BDH Chemicals Ltd., Poole, England
Molecular sieve (Potassium, Sodium alumino-silicate) (nominal pore diameter: 3 angstrom)	/	Sigma Chemical Co., St. Louis, MO, USA
MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; thiazolyl blue) ($C_{18}H_{16}N_5SBr$)	414.3	Sigma Chemical Co., St. Louis, MO, USA
Non-fat milk	/	Carnation, Nestle
Penicillin-streptomycin (penicillin: 5000 U/ml protein streptomycin: 5000 g/ml)	/	GibcoBRL, Life Technologies Inc.
Phenol (C_6H_5OH)	94.11	USB (United States Biochemical), Cleveland, OH, USA
Phosphoric acid (H_3PO_4)	/	Sigma Chemical Co., St. Louis, MO, USA
PIPES (piperazine-N,N'-*bis*(2-ethanesulfonic Acid))	302.4	Boehringer Mannheim, Germany
PMSF (phenylmethyl-sulfonyl fluoride) ($C_7H_7FO_2S$)	174.2	Sigma Chemical Co., St. Louis, MO, USA
Polyvinylpyrrolidone (PVP)	40,000	Sigma Chemical Co., St. Louis, MO, USA
Potassium chloride (KCl)	74.55	Sigma Chemical Co., St. Louis, MO, USA
RPMI (with phenol red)	/	GibcoBRL, Life Technologies Inc.
RPMI (without phenol red)	/	GibcoBRL, Life Technologies Inc.
SDS (sodium dodecyl sulfate) ($CH_3(CH_2)_{11}SO_4Na$)	288.38	USB (United States Biochemical), Cleveland, OH, USA
Sodium chloride (NaCl)	58.44	USB (United States Biochemical), Cleveland, OH, USA
Sodium citrate ($C_6H_5Na_3O_7 \cdot 2H_2O$)	294.1	Sigma Chemical Co., St. Louis, MO, USA
Sodium dihydrogen phosphate (NaH_2PO_4)	156.01	Riedel-de Haen, AG, Germany

Sodium hydrogen phosphate (Na_2HPO_4)	141.96	Sigma Chemical Co., St. Louis, MO, USA
Sodium hydroxide (NaOH)	40.00	Sigma Chemical Co., St. Louis, MO, USA
Sodium orthovanadate (Na_3VO_4)	183.9	Sigma Chemical Co., St. Louis, MO, USA
Sorbitol (D-Glucitol) ($C_6H_{14}O_6$)	182.2	Sigma Chemical Co., St. Louis, MO, USA
Sucrose ($C_{12}H_{22}O_{11}$)	342.3	Sigma Chemical Co., St. Louis, MO, USA
***Taq* DNA polymerase** (with 10X PCR buffer and 25 mM $MgCl_2$)	/	GibcoBRL, Life Technologies Inc. or MBI, Fermantas
TEMED (N, N, N', N'-tetramethyl-ethylene diamine) ($(CH_3)_2NCH_2CH_2N(CH_3)_2$)	116.21	Boehringer Mannheim, Germany
TNF-α (recombinant murine tumor necrosis factor-alpha)	/	Roche Diagnostics
Tris ($NH_2C(CH_2OH)_3$)	121.14	USB (United States Biochemical), Cleveland, OH, USA
Trypsin-EDTA (0.25% trypsin, 1 mM EDTA)	/	GibcoBRL, Life Technologies Inc.
Tween-20 (polyoxyethylene-sorbitan monolaurate)	/	Sigma Chemical Co., St. Louis, MO, USA
X-Gal 5-Bromo-4-chloro-3-indolyl-b-D-galactopyranoside ($C_{14}H_{15}BrClNO_6$)	408.6	USB (United States Biochemical), Cleveland, OH, USA
Xylene cyanol FF ($C_{25}H_{27}N_2O_6S_2Na$)	538.6	Amersham Pharmacia Biotech

Cytochrome *c*

Cytochrome *c* from different species were purchased from Sigma in powder form and stored at 4 °C. It is dissolved in distilled water, binding buffer or PBS according to the usage and stored at -20 °C.

Recombinant Murine TNF-α

Recombinant murine TNF-α is produced by *E. coli* and purified by standard chromatographic techniques and has more than 95% purity as determined by SDS-PAGE (Manual of Roche Diagnostics). It was dissolved in PBS with the stock

concentration of 5 μg/ml and was stored at -70 °C with minimal repeated freezing and thawing. It has the specific activity of more than 6.0×10^7 U/mg. The unit definition is that one unit is defined as the amount of TNF-α required for mediating half-maximal cytotoxicity with L929 cells in the presence of actinomycin D.

Antibodies for Western Blotting

Primary antibody used was anti-cytochrome *c* (1:2000, recognizes 15 kDa protein) (clone number 7H8.2C12, Pharmingen). It was kept at 4 °C and repeated freezing and thawing were avoided. When the antibody was in use, it was diluted in 5% (w/v) non-fat milk or 1% (w/v) BSA in TBS-T.

Other Chemicals

Aprotinin, leupeptin, PMSF, ammonium persulfate, agarose, proteinase K, RNase A, ethidium bromide were used. Aprotinin and leupeptin were dissolved in dH_2O and stored at -20 °C. PMSF was dissolved in absolute ethanol and stored at -20 °C. Ammonium persulfate was dissolved in dH_2O and stored at 4 °C. Agarose was dissolved in TBE buffer. Ethidium bromide solution was diluted with dH_2O and kept at room temperature in dark. Proteinase K and RNase A were dissolved in dH_2O and TE buffer respectively and kept at -20 °C.

2.1.2. Buffers

Buffer was prepared by dissolving chemicals in distilled water (dH_2O) and titrated to suitable pH with either HCl or NaOH, unless otherwise specified.

2.1.2.1. Buffers commonly used

Binding Buffer was prepared with 10 mM HEPES, 75 mM NaCl, 5 mM $MgCl_2$, 1 mM $CaCl_2$. The pH was then adjusted to 7.4 and the buffer was stored at room temperature.

Elution Buffer was prepared with 20 mM Tris-acetate, 4 M guanidine thiocyanate. The pH was then adjusted to 8.3 and the buffer was stored at room temperature, 1 mM DTT was added before used.

Phosphate buffered saline (PBS) was prepared using 2.7 mM KCl, 1.5 mM KH_2PO_4, 136 mM NaCl and 8 mM Na_2HPO_4 and the pH was adjusted to 7.4. PBS was made sterilized by autoclave and was then stored at 4 °C.

2.1.2.2. Reagent for molecular work

6X loading buffer was prepared with 0.25% (w/v) bromophenol blue, 0.25% (w/v) xylene cyanol FF and 40% (w/v) sucrose and the buffer was stored at 4 °C.

TE buffer was prepared with 10 mM Tris and 0.5 mM EDTA. The pH was then adjusted to 7.4. The buffer was sterilized by autoclave and was then stored at room temperature.

5X TBE buffer was prepared form the pre-weighed and pre-mixed powder purchased from USB. The powder was reconstituted to a 5X TBE solution by adding dH_2O to 400 ml. After reconstitution, the 5X solution is: 0.445 M Tris, 0.445 M boric aid and 0.01 M EDTA·Na_4. The 5X TBE buffer was stored at 4°C.

2.1.3. Bacterial culture

Medium of bacterial culture

LB and SOB were purchased in pre-mixed powder form. After dissolved in distilled water and autoclaved, the medium were stored at 4 °C. Suitable antibiotic was added before used.

LB plate

LB plate was prepared by 20 g/L LB, 1.5% agar in distilled water. After autoclaving and cooling to 55 °C, ampicillin was added to a final concentration of 50 μg/ml. Plates were then filled and stored at 4 °C until use.

2.1.4. Culture of cell

2.1.4.1. TNF-α Sensitive Cell Line, L929

L929 cells were purchased from American Type Culture Collection (ATCC, Rockville, MD, USA) and they are TNF-α sensitive cell line. They were established from the 95th subculture generation of the parent L strain of fibroblasts in the C3H/An mouse. They have adherent characteristics and were maintained in RPMI 1640 medium supplemented with 10% (v/v) fetal bovine serum and 1% penicillin-streptomycin (v/v) (complete medium) at 37 °C, 5% CO_2 incubator (SHEL LAB) with humidified atmosphere.

2.1.4.2. Medium of cell culture

RPMI 1640 (Phenol Red Medium)

RPMI (Rosewell Park Memorial Institute) 1640 was the medium used for culturing of TNF-α sensitive cell line. It consisted of phenol red, L-glutamine and 0.5

mM HEPES. The power of RPMI was then dissolved in one liter of dH_2O and was supplemented with 24 mM $NaHCO_3$. The pH of the medium was adjusted to 7.4. Then, the medium was sterile filtered with a 0.22 μm cellulose acetate membrane bottle-top filter (7111, Falcon, Becton Dickinson, NJ, USA). The medium was then supplemented with 10% FBS (v/v) and 1% penicillin-streptomycin (v/v) for cell culture. The medium was stored at 4 °C.

RPMI 1640 (Phenol Red-free Medium)

Phenol red-free RPMI medium consisted of L-glutamine without phenol red or HEPES. The powder of phenol red-free RPMI was then dissolved in one liter of dH_2O and was supplemented with 24 mM $NaHCO_3$ and 25 mM HEPES. The pH of the medium was adjusted to 7.4. Then, the medium was sterile filtered with a 0.22 μm cellulose acetate membrane bottle-top filter. The colorless medium was supplemented with 1% penicillin-streptomycin (v/v) only. The medium was then stored at 4 °C.

2.1.5. Reagent for Western blotting

2.1.5.1. Protein extraction

Lysis buffer for total protein extraction was prepared with 1 mM Na_3VO_4, 1% (w/v) SDS, 10 mM Tris. The pH was then adjusted to 7.4 and stored at room temperature. Prior to the lysis of cells, the lysis buffer was supplemented with 5 mM $MgCl_2$, 1 mM PMSF and protease inhibitors such as 21 μg/ml aprotinin and 5 μg/ml leupeptin.

Lysis buffer for cytochrome *c* extraction was prepared with 75 mM NaCl, 1 mM NaH_2PO_4, 8 mM Na_2HPO_4, 250 mM sucrose. The buffer was stored at 4 °C. Prior

to the lysis of cells, the buffer was supplemented with 1 mM PMSF, 21 μg/ml aprotinin, 5 μg/ml leupeptin and various concentrations of digitonin.

2.1.5.2. SDS-PAGE

4X lower gel buffer was prepared with 1.5 M Tris base and 14 mM SDS. The pH of the buffer was adjusted to 8.8 and stored at 4 °C.

4X upper gel buffer was prepared with 0.5 M Tris base and 14 mM SDS. The pH of the buffer was adjusted to 6.8 and stored at 4 °C.

15% separating gel buffer was freshly prepared with 50% (v/v) acrylamide/bis-acrylamide (30%) solution, 25% (v/v) 4X lower gel buffer, 0.05% (w/v) ammonium persulfate and 0.12% (v/v) TEMED.

4.5% stacking gel buffer was freshly prepared with 15% (v/v) acrylamide/bis-acrylamide (30%) solution, 25% (v/v) 4X upper gel buffer, 0.07% (w/v) ammonium persulfate and 0.13% (v/v) TEMED.

2X SDS loading buffer was prepared with 2% (w/v) SDS, 10% (w/v) sucrose, 0.002% bromophenol blue and 62.5 mM Tris. The pH of the buffer was adjusted to 6.8. Buffer was supplemented with 5% (v/v) 2-mercaptoethanol and stored at 4 °C.

SDS tank buffer was prepared with 25 mM Tris base, 192 mM glycine and 0.1% (w/v) SDS and stored at room temperature.

Commassie Blue staining solution for protein staining was prepared with acetic acid, methanol and dH_2O with a ratio of 1:3:10. 0.05% (w/v) commassie brilliant blue R-250 was added and the solution was stored at room temperature.

De-stain solution for removing commassie brilliant blue R-250 was prepared with 40% (v/v) methanol and 10% (v/v) acetic acid and was stored at room temperature.

2.1.5.3. Electro-blotting

Electroblot buffer was freshly prepared with 48 mM Tris base, 39 mM glycine, 20% (v/v) methanol and 0.0375% (w/v) SDS. The pH of the buffer was adjusted to 9.2 to 9.4.

TBS-Tween-20 (TBS-T) was prepared with 10 mM Tris base, 0.15 M NaCl and 0.1% (v/v) Tween-20 and stored at room temperature.

2.2. Methods

2.2.1. Conjugation of protein to solid support

2.2.1.1. Conjugation of protein on PVDF membrane

The dry 0.22 μm PVDF (polyvinylidene fluoride) membrane (Immobilon-P^{SQ}, Millipore) was rehydrated with absolute methanol and then immersed in PBS for 15 min. After removing the excess PBS by kimwipes, the membrane was placed on kimwipes. Then cytochrome *c* in PBS was dropped on the membrane and the cytochrome *c* was conjugated on the membrane after the solution was soaked through the membrane.

2.2.1.2. Conjugation of protein on Sepharose

N-Hydroxysuccinimidyl-Agarose (Sigma) was used as solid support and was first packed in column. The isopropanol as stock buffer of the Sepharose was washed out with 1 mM ice-cold HCl and then 5 mg/ml cytochrome *c* in coupling buffer (0.2 M $NaHCO_3$, 0.5 M NaCl, pH 8.3) was loaded and incubated for 30 minutes at room temperature. After that, the uncoupled cytochrome *c* was washed away with the coupling buffer and the excess active groups of the solid support that have not coupled to the ligand were deactivated by buffer A (0.5 M ethanolamine, 0.5 M NaCl, pH 8.3) and buffer B (0.1 M acetate, 0.5 M NaCl, pH 4.0) alternatively for 3 cycles. The column was finally washed with PBS and stored at 4 °C in PBS.

2.2.1.3. Conjugation of protein on magnetic bead

Before conjugation of cytochrome *c*, the magnetic beads (Dynabeads M-280 Tosylactivated) were resuspended well and washed once with Buffer A (0.1 M Na-phosphate buffer, pH 7.4, 0.019 M Na H_2PO_4 and 0.081 M Na_2 HPO_4). Then 3 μg

cytochrome *c* was added per 10^7 magnetic beads (approximately 20 μg/mg). After resuspended the Dynabeads thoroughly, a final concentration of 1-2 x 10^9 beads per ml coating solution was resulted. The coating mixture was incubated for 16-24 hr at 37 °C with slow tilt rotation.

After incubation, the supernatant was removed and the magnet beads were collected. The beads were then washed four times with two times in Buffer C (PBS, pH 7.4 with 0.1% (w/v) BSA) for 5 minutes at 4 °C, once in Buffer D (0.2 M Tris, pH 8.5 with 0.1% (w/v) BSA) for 24 hr at 20 °C or for 4 hr at 37 °C and the last time in Buffer C for 5 minutes at 4 °C. Then the magnetic beads were cytochrome *c* conjugated and were ready for use. The unused cytochrome *c* conjugated beads were stored in Buffer C at 4 °C.

2.2.2. SELEX

2.2.2.1. Selection

Before selection, the oligonucleotide pool was first heated in binding buffer at 95 °C for 3 minutes. And then, the heated oilgonucleotide pool was incubated with the protein conjugated solid support in binding buffer for various times at various temperatures as indicated. Then the solid support was collected and washed with binding buffer for several times. The unbound oligonucleotides were washed away and the bound oligonucleotides were then eluted with elution buffer or by excess ligand in binding buffer. The elute collected was then extracted with phenol: isoamyl alcohol in equal volume and precipitated with 100% ethanol in the presence of 1/10 volume 3 M sodium acetate (pH 5.5) and 25 μg of glycogen at -20 °C. The precipitate was collected with centrifugation at 20,812 × *g* (14,000 rpm, 9.5 cm, Eppendorf

Centrifuge 5417R) for 20 mins at 4 °C. The pellet collect was then washed with ice-cold 70% ethanol and another centrifugation at 20,812 × *g* (14,000 rpm, 9.5 cm, Eppendorf Centrifuge 5417R) for 20 mins at 4 °C. The precipitate was then dried in air or oven and then dissolved in autoclaved distilled H_2O.

2.2.2.2. Photo-selection

Production of Photo-oligonucleotide pool

PCR was done to incorporate dBrUTP into the oligonucletoides. In 1.5 ml reaction in standard *Taq* PCR buffer including 1.5 mM $MgCl_2$, 30 pmol templates was added with 200 μM each of dATP, dGTP, dCTP and dBrUTP and 300 nM of forward and reverse primers which are complementary to the flanked end of the oligonucleotides. The reaction mixture was heated at 95 °C for 3 minutes before adding 18.75 units of *Taq* and 18.75 units of Pwo. After enzymes were added, PCR was preformed for 16 cycles of 95 °C for 40 secs, 55 °C for 1 min and 72 °C for 2 mins and finally terminated with 72 °C for 10 mins.

The PCR product was then concentrated by using Microcon YM-30, regenerated cellulose 30,000 MWCO (Amicon, Millipore Corporation, Bedford, MA) and eluted with 50 μl dH_2O. Polyacrylamide (12%) with 7 M urea denaturing gel was then run to confirm the size and also purify the photo-oligonucleotide pool from the excess deoxynucleotide and the enzymes.

The band in suitable size was cut and then crashed. Binding buffer was added and incubated with the crashed gel at 37 °C overnight with rotation. The solution was then separated from the gel by using multispin separation column (Axygen ™

Scientific, California, USA) and absorbance at 260 nm was measured to qualify the amount of oligonucleotide pool in the solution.

Photo selection

The photo-oligonucleotide pool in final concentration of 50 nM was incubated with target ligand in a final concentration of 1.5 nM in a total volume of 800 µl for 15 mins at room temperature with gently shaking. After incubation, the solution was irritated by UV light from UV / White light transilluminator (Spectroline, model TVD-1000R) for 2 mins. The solution was then collected and filtered by using Microcon YM-30, regenerated cellulose 30,000 MWCO (Amicon, Millipore Corporation, Bedford, MA). All the bound aptamer-ligand complex, free ligand and free oligonucleotide remained on the filter and were then eluted by dH_2O. The eluate was then mixed with 0.2 volume of 6X loading buffer and 0.2 volume of 7M urea and heated at 95 °C for 3 minutes. The bound and unbound oilgonucleotides were then separated by 12% polyacrylamide with 7 M urea denaturing gel. After staining with ethidium bromide and visualized by UV light, the band run slower in the gel were cut and crashed. TE buffer with 400 µg/ml proteinase K was added and incubated with the crashed gel at 37 °C overnight with rotating. The eluted aptamer were then collected and extracted with phenol:chromoform. After precipitated with ethanol, aptamer was dissolved in dH_2O and amplified by PCR.

2.2.2.3. PCR

PCR was performed with the aptamer selected. Various amount of aptamer was gently mixed with 2.5 µl of 10X PCR buffer (final conc. of 1X), 1.5 µl of 25 mM $MgCl_2$ (final conc. of 1.5 mM), 0.5 µl of 10 mM dNTP mix (final conc. of 0.2 mM),

0.5μl of 300 μM forward primer (for single strand PCR, final conc. of 6 μM) or 10 μM forward primer (for double stand PCR, final conc. of 0.2 μM) and 0.5 μl of 10 μM of reverse primer (final conc. of 0.2 μM), 0.25 μl of *Taq* DNA polymerase (5 units/μl, final conc. of 1.25 units/25 μl) and added up the total volume to 25 μl with autoclaved dH_2O. PCR was then performed with the following conditions.

Table 2.2 Summary of PCR condition.

Conditions	Temperature	Time	Number of cycles
Initial denaturation	95 °C	3 min	1
Denaturation	95 °C	45 sec	various number of cycles
Annealing	55 °C	30 sec	
Elongation	72 °C	1 min 30 sec	
Final elongation	72 °C	10 min	1

2.2.3. Separation of oligonucleotides

2.2.3.1. Separate short length double-stranded oligonucleotides by using polyacrylamide gel

The apparatus of Mini-Protean II cell (BIO-RAD Laboratories) for SDS-PAGE was set up according to the manual.

Polyacrylamide (12%) separating gel in 0.5X TBE buffer was used. For each sample, 1 volume of DNA sample was mixed with 1/5 volume of 6X DNA loading buffer. Then, DNA samples, together with the suitable DNA markers were loaded on each well. The voltage was set at 200 V and protein samples were run for about 45 minutes. After that, gels were removed from the apparatus and were soaked in ethidium bromide.

Fig. 2.1. Double-stranded PCR products separated by native polyacrylamide gel.

After PCR amplification, the products were mixed with 1/5 volume of 6X DNA loading buffer and then loaded into wells. After separation by 12% polyacrylamide gel, DNA was stained with ethidium bromide and visualized under UV light. DNA ladder (25 bp, 0.4 μg) was used as size marker.

2.2.3.2. Separate short length single-stranded oligonucleotides by using denaturing polyacrylamide gel

The apparatus of Mini-Protean II cell (BIO-RAD Laboratories) for SDS-PAGE was set up according to the manual.

To separate single-stranded DNA, 12% polyacrylamide with 7M urea separating gel in 0.5X TBE was used. For each sample, 1 volume of DNA sample was mixed with 1/4 volume of 6X DNA loading buffer and 1/4 volume of 7M urea. Then the mixture was heated at 95°C for 3 minutes. DNA samples, together with the suitable DNA markers were loaded on each well. The voltage was set at 200 V and DNA samples were run for about 45 minutes. After that, gels were removed from the apparatus and were soaked in ethidium bromide.

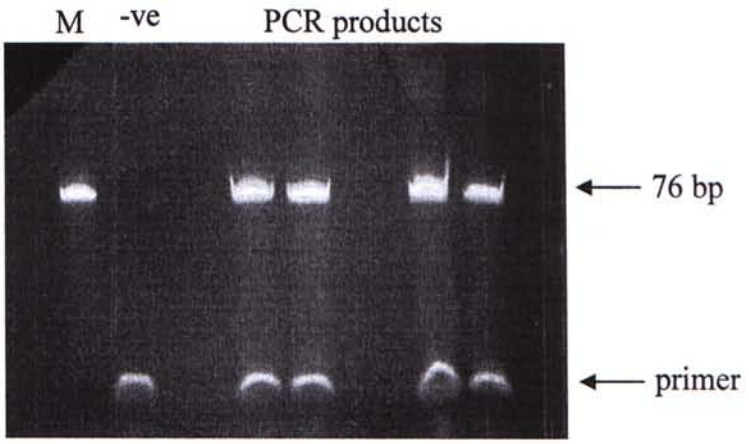


Fig. 2.2. Single-stranded PCR products separated by denaturing polyacrylamide gel.

After PCR amplification, the products were mixed with 1/4 volume of 6X DNA loading buffer and 1/4 volume of 7M urea. The mixtures were heated at 95 °C for 3 minutes and then loaded into wells. After separation by 12% polyacrylamide gel with 7M urea, the DNA were stained with ethidium bromide and visualized under UV light. Ten pmol of pool of the oligonucleotides were used as marker (M). PCR amplification without any oligonucleotides added (-ve).

2.2.3.3. Extract the DNA from polyacrylamide gel

After cutting the band containing the suitable size of DNA, the gel was stored in a pre-weighed microfuge tube. The gel band with the tube was then weighed and the net weight of the gel band was then calculated. After dring the gel band in oven, it was then crashed into powder. Binding buffer in 2X volume of the gel band was added to the crashed gel and incubated at 37 °C for overnight or 70 °C for 3 hours.

The solution with DNA eluted was then separated from the gel by using multi spin separation column (Axygen $_{TM}$ Scientific, California, USA) and the amount of

oligonucleotide pool in the solution was then qualified by measuring the absorbance at 260 nm.

2.2.3.4. Estimate the amount of DNA in solution after extraction

The amount of DNA in solution was estimated by measuring absorbance at 260 nm. For $O.D._{260} = 1$, there should be 33 μg single stranded DNA or 50 μg double strand DNA in 1 ml solution.

$$\text{Concentration of single stranded DNA (mol/μl)} = \frac{O.D.260nm \times 33 \times 10^{-6}}{Molecularweightof the DNA} \div 1000$$

$$\text{Concentration of double stranded DNA (mol/μl)} = \frac{O.D.260nm \times 50 \times 10^{-6}}{Molecularweightof the DNA} \div 1000$$

2.2.3.5. Agarose Gel Electrophoresis

Agarose gel electrophoresis is used to separate DNA and RNA according to their molecular weight under electric field.

One percent agarose gel was prepared by dissolving the agarose powder in 1X TBE buffer in the flask. The flask was then weighed and was put into the microwave oven to heat for 1 min until the agarose dissolved. The mixture was then cooled to around 50 °C and was poured into the cassette until the agarose formed the gel like structure.

The DNA was then run in agarose gel at constant voltage. After electrophoresis,

the gel was stained with ethidium bromide for 10-20 min, followed by destaining in dH_2O for another 10-20 min. The gel was then put onto the UV / White light transilluminator (Spectroline, model TVD-1000R).

2.2.4. Cloning of selected polyclonal aptamer

2.2.4.1. Restriction cutting

For restriction cutting of the plasmid or aptamer, 1 μg of DNA was mixed with 1 μl restriction enzyme, in a final volume of 20 μl of 1X restriction buffer. The reaction was preformed at 37 °C for 2 hours and heat inactivated at 65 °C or 80 °C for 20 minutes.

2.2.4.2. Ligation

In a 10 μl ligation reaction system, it consists of 1 μl T4 ligase, 1 μl 10X ligase buffer and a total of 500 ng DNA. The vector and insert were in 1:4 ratios. The reaction was preformed at 16 °C overnight.

2.2.4.3. Preparation of competent cells

Bacterial cell was first incubated in 2 ml LB with shaking at 37 °C overnight. After that, the bacterial culture was added to 100 ml LB medium and shaking at 37 °C until absorbance at 550 nm reached 0.48. The bacterial culture was then iced for 15 minutes and centrifuged at 4,000 g for 5 minutes. 0.4 volume of Tfb I solution (30 mM potassium acetate, 100 mM rubidium chloride, 10 mM calcium chloride, 50 mM manganese chloride, 15% (v/v) glycerol, pH 5.8, with dilute acetic acid) was added to resuspend the pellet. The suspension was then iced for another 15 minutes and centrifuged at 4,000 g for 5 minutes. 0.04 volume of Tfb II (10 mM MOPS, 75 mM

calcium chloride, 10 mM rubidium chloride, 15% glycerol, pH 6.5, with dilute sodium chloride) was added to resuspend the pellet. After keeping the bacteria in an ice bath for 15 minutes, the competent cells can be used immediately or quick freeze at -70 °C. The competent cells should be stored in small amount per tube and thaw and freeze should be avoided.

2.2.4.4. Transformation of plasmid into competent cell

At the bottom of a cap tube in ice, 5 µl of the ligation product was mixed with 50 µl competent cells. The tube was then kept in ice for 30 minutes and was transferred to a water bath per-heated at 43 °C for exactly 90 seconds. After heat shocked, the tube was then placed in ice for another 2 minutes. Pre warmed LB (1 ml) was then added to the tube and the tube was warmed in a 37 °C water bath for 45 – 60 minutes. The bacterial culture was then centrifuged at 5,000 r.p.m. for 5 minutes. The supernatant was discarded and 100 µl of LB was added to resuspend the pellet. All the bacteria were transferred to and spread evenly on a LB plate with suitable antibiotics. The plate was then incubated at 37 °C overnight.

2.2.4.5. Plasmid extraction from bacterial culture

Bacteria colony was picked and incubated in 2 ml of LB with suitable antibiotics at 37 °C overnight with shaking. 1.5 ml bacterial culture was transferred to a microfuge tube and centrifuged at 20,800 × *g* (14,000 rpm, 9.5 cm, Eppendorf Centrifuge 5417R) for 30 seconds. The supernatant was then removed thoroughly. One hundred µl ice-cold Alkaline Lysis Solution I was then added to resuspend the pellet. After vigorous vortexing, 200 µl freshly prepared Alkaline Lysis Solution II was added and mixed thoroughly without vortexing. Alkaline Lysis Solution (150 µl)

III was then added. The tubes were inverted several times and stored in ice for 5 minutes. The lysate was then centrifuged at 20,800 × *g* (14,000 rpm, 9.5 cm, Eppendorf Centrifuge 5417R) for 5 minutes at 4 °C. The upper layer was then transferred to a new microfuge tube and extracted once with phenol:chloroform and once with chloroform. The plasmid was then precipitated by ethanol and washed once by 70% ethanol. The pellet was then dried and dissolved in 50 µl TE buffer.

2.2.5. Cell culture

2.2.5.1. Cell culture of L929

Generally, L929 cells were kept in 25, 75 or 125 cm^3 culture flasks and passages were made every 3 or 4 days after growing to confluence. In each passage, the spent medium was discarded. Cells were then washed with PBS once and trypsinized from the culture flask. Complete medium was then added and cells were collected. Centrifugation was carried out at 453 × *g* (1,500 rpm, 16.0 cm, Eppendorf Centrifuge 5810R) for 3 min. Cells pellet was then resuspended in complete medium and transferred to a new culture flask.

2.2.5.2. Preservation of cells

Frozen stock of cells were kept in freezing mixture and stored in liquid nitrogen. Cells trypsinized from culture flasks were resuspended with complete medium. Centrifugation was then carried out at 453 × *g* (1,500 rpm, 16.0 cm, Eppendorf Centrifuge 5810R) for 3 min. Cells (5×10^6) were resuspended in one ml freezing mixture. The freezing mixture composed of 50% FBS (v/v), 40% complete medium (v/v) and 10% DMSO (v/v). Cell suspension (1 ml) was transferred to the freezing vials. The cells were then cooled to -20 °C until frozen and vials of cells were then

kept at -70 °C overnight. Afterwards, cells were stored in liquid nitrogen until use.

To thaw the cells, cells were thawed quickly and diluted in excess pre-warmed complete medium. Centrifugation at 453 × *g* (1,500 rpm, 16.0 cm, Eppendorf Centrifuge 5810R) for 3 min was then carried out and pellet of cells was suspended into complete medium and cells were transferred to a new culture flask.

2.2.5.3. Treatment with TNF-α

Cells (1 x 10^6/ml) in complete medium were seeded on 6-well plates at 37 °C and 5% CO_2 overnight. On the day of experiment, cells were washed with serum-free medium twice. Cells were then treated with TNF-α (50 ng/ml) for various time intervals. Control cells were only treated with serum-free medium alone. After treatment, cells were collected for different assays.

2.2.5.4. Fixation of cell

L929 cells (2.5 x 10^5) were seeded in 24-wells plates in complete medium and incubated at 37 °C and 5% CO_2 overnight. The cells were then washed with PBS twice. Glutaraldehyde (0.3%) in PBS (1 ml) was added to each well and incubated for 10 minutes at room temperature. The cells were then washed with PBS two more times. Triton X-100 (1%) in PBS (1 ml) was then added and mixture was incubated for 15 minutes at room temperature. After washed twice with PBS, non-specific blocking was done by incubating the cells with 1% BSA in PBS at 4 °C overnight. On the next day, the cells were washed with PBS for two more times and then stored in PBS at 4 °C until used.

2.2.6. Western blotting analysis

2.2.6.1. Preparation of proteins from cells

Cellular proteins can be isolated with the use of digitonin of various concentrations. L929 cells with appropriate treatments were washed with serum-free medium twice and then trypsinized. After centrifugation, the cell pellet was washed with serum-free medium twice and the number of cells was then counted. In each sample, 50 μl of lysis buffer (75 mM NaCl, 1 mM NaH_2PO_4, 8 mM Na_2HPO_4, 250 mM sucrose, 21 μg/ml aprotinin, 5 μg/ml leupeptin, 1 mM PMSF and various concentrations of digitonin) was added to 4 x 10^6 cells and the mixture was vortex vigorously for 30 seconds at room temperature. After that, samples were centrifuged at 10,621 × *g* (10,000 rpm, 9.5 cm, Eppendorf Centrifuge 5417R) for 1 min at room temperature with a centrifuge (MSE, MicroCentaur, Sanyo). Subsequently, supernatant was then collected and 50 μl of 2X SDS loading buffer was added. The mixture was then boiled for 10 min and centrifugation was then carried out at 20,817 × *g* (14,000 rpm, 9.5 cm, Eppendorf Centrifuge 5417R) at 4 °C for 7 min. After that, the supernatant was collected and the cell lysate was stored at -70 °C.

2.2.6.2. SDS polyacrylamide gel electrophoresis (SDS-PAGE)

The apparatus of Mini-Protean II cell (BIO-RAD Laboratories) for SDS-PAGE was set up according to the instruction.

SDS polyacrylamide separating gel (15%) and 4.5% stacking gel were used. In each experiment, 20 μl of cell lysate was boiled for 5 min before used. Then, protein samples, together with the low range protein marker were loaded on each well. The voltage was set at 150 V and protein samples were run for 1.25 hr. After that, gels

were removed from the apparatus and were soaked in electroblot buffer.

2.2.6.3. Electroblotting of protein

The electroblotting of proteins was carried out with the use of the Mini Trans-Blot Cell (tank transfer system, BIO-RAD Laboratories).

Prior to the electroblotting, the dry 0.22 μm PVDF (polyvinylidene fluoride) membrane (Immobilon-P^{SQ}, Millipore) was rehydrated with absolute methanol and then immersed in electroblot buffer for 15 minutes. Moreover, 1 mm filter paper (Whatman) was also soaked in electroblot buffer before use. The tank transfer system for electroblotting was set up with the following sequence: cathode, fiber pad, 3x filter paper, gel, PVDF membrane, 3x filter paper, fiber pad and then anode. Electroblotting was then performed with the voltage of 100 V for 1 hr.

After electroblotting, the gels were stained with commassie blue staining solution overnight for protein determination and then de-stained with de-stain solution for another day.

2.2.6.4. Probing antibodies or aptamers for proteins

After blotting, the membrane was transferred to a petri dish and 20 ml of 1% (w/v) BSA or 5% (w/v) non-fat milk in TBS-T or binding buffer was added and incubated at 4 °C overnight for non-specific blocking. After that, the membrane was washed with TBS-T or binding buffer twice for 15 min. The membrane was probed with primary antibody or aptamer for 1 hr at room temperature with rotations. The antibody or aptamer was then removed and the membrane was washed with TBS-T or

binding buffer twice for 15 min for three times. HRP conjugated anti-mouse antibody or streptavidin HRP conjugated antibody was then loaded to the membrane for another hour. After that, the membrane was washed with TBS-T or binding buffer for 15 mins for three times also.

2.2.6.5. Enhanced chemiluminescence (ECL) Assay

ECL assay was performed by mixing the ECL reagent 1 and reagent 2 in a ratio of 1:1 (Amersham Pharmacia Biotech). The membrane was then exposed to the ECL reagent mixture for 1 min at room temperature. After that, the membrane was wrapped with Saran wrap and was developed on Fuji Medical x-ray film (super Rx, Fuji) at various time periods. Films were then processed in a film processor (M35 X-OMAT, Kodak).

Chapter 3
Results

In this project, we were trying to select an aptamer that can bind cytochrome *c* specifically with high affinity. To start the selection, 3-APT, a pool of oligonucleotides containing 40 random nucleotide sequences was used.

3.1 Selection of aptamer against cytochrome *c* dotted on membrane with counter selection against BSA also on membrane.

3.1.1 Selection process

To start the first selection, 100 pmol of the oligonucleotides were used. The oligonucleotides were heated at 95 °C for 3 minutes in binding buffer to unfold their secondary and tertiary structures and let them refold into their unique sequence-dependent structures. After refolding, the pool was first counter selected against 1% (w/v) BSA coated on a PVDF membrane. By this counter selection, oligonucleotides that bind to the solid support or the blocking agent BSA were eliminated. Oligonucleotides remained were then selected against cytochrome *c* (1 μg) coated on another PVDF membrane. After several rounds of washing with binding buffer, the oligonucleotides bound to the target were eluted by the elution buffer at 90 °C for 10 minutes. The oligonucleotides were then extracted with phenol: chloroform: isoamyl alcohol (v:v:v = 25:24:1) and precipitated with ethanol. The oligonucleotides collected were then amplified by PCR with primer pair which is complementary to the flanked ends of the oligonucleotides. The amount of 5' forward primer was 30 times more than that of the 3' reverse primer to produce more the single-stranded sense oligonucleotides. The PCR products were then subject to the second round of selection.

3.1.1.1 PCR cycles

After the selection of oligonucleotides against cytochrome *c*, oligonucleotides were amplified by PCR. Before starting the PCR, the optimal number of amplification should be determined. Theoretically, the PCR cycle number is mostly restricted in the range of 15-25. This is because after the optimal number of cycles of PCR, product of the suitable size will be saturated and high molecular PCR products will be further amplified during the excess PCR cycles. To eliminate this artifact, the optimal cycle number for PCR was investigated.

In a final 50 μl PCR reaction mixture, 1 pmol of oligonucleotides was added and PCR performed. Started from cycle 10, 10 μl of the reaction mixture was collected for every 5 cycles until cycle 30. The reaction mixtures were then separated by a 12% polyacrylamide 7 M urea denaturing gel (Fig. 3.1).

From the results, it can be seen that the amount of high molecular weight product increased with the number of PCR cycle. However, the intensity of the band of oligonucleotides with the size of 76 bp did not increase much when the cycle number was beyond 15. These results suggest that 15 cycles was the optimal number for this PCR amplification condition (Fig. 3.1).

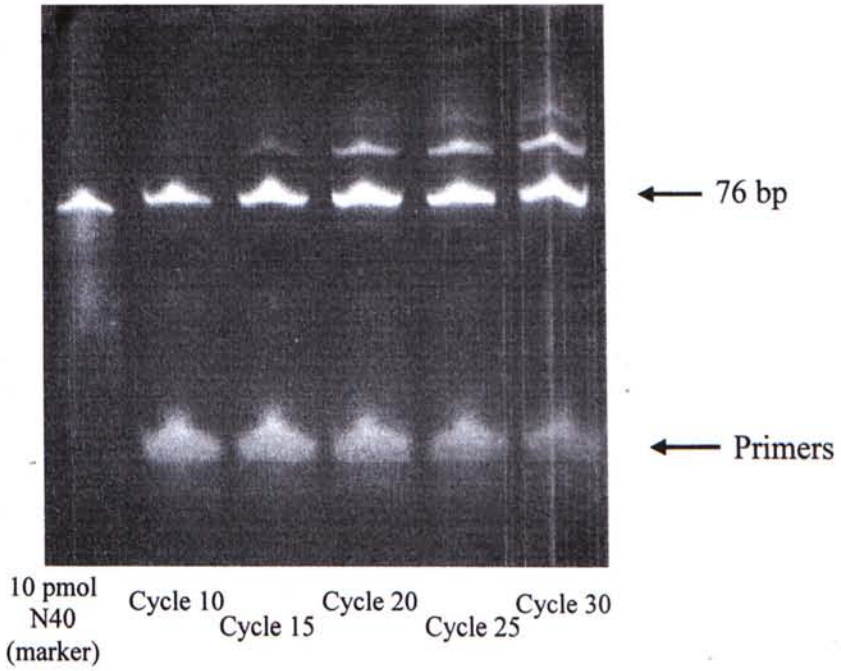


Fig. 3.1. A denaturing gel showing the PCR products from different cycle numbers.

Oligonucleotides (1 pmol) were added to a PCR reaction mixture (final volume 25 μl). Thirty cycles of amplification were performed. Ten μl of the PCR products were collected from the mixture after cycle 10, 15, 20, 25, and 30. The products were then mixed with 2.5 μl urea (7M) and 2.5 μl 6X loading buffer. After heated at 95 °C for 3 minutes, the mixtures and 10 pmol oligonucleotides of size 76 bp were loaded to the well of 12% polyacrylamide with 7 M urea denaturing gel as indicated. After separation, DNA bands were visualized under UV after staining with ethidium bromide.

3.1.1.2 Polyclonal aptamer

After several rounds of selection, the selected aptamers should be polyclonal (i.e. carry different sequences in the core fragment). They were first tested by dot blot analysis. Various amounts of cytochrome *c* were dotted on PVDF membrane and non-specific blocking was done by 1% (w/v) BSA. The cytochrome *c* on the membrane were then labeled with the aptamers selected. To generate signals, the aptamers selected were amplified by PCR with excess biotinylated 5' forward primer in the last round of amplification. The single stranded sense aptamers so produced were biotinylated which could be recognized by streptavidin. After labeling and washing, streptavidin with HRP conjugated and suitable substrates were added and bioluminescent signals were generated and detected by X-ray films. Our results indicate that streptavidin HRP at 5000-fold dilution was too strong (i.e. too dark) while dilution of 10000-fold was the optimal concentration (data not shown).

Results below showed that the polyclonal aptamers selected could label cytochrome *c* in a dose-dependent manner (Fig. 3.2). In this Western blot, 0.5 μg of cytochrome *c* seemed to be the detection limit.

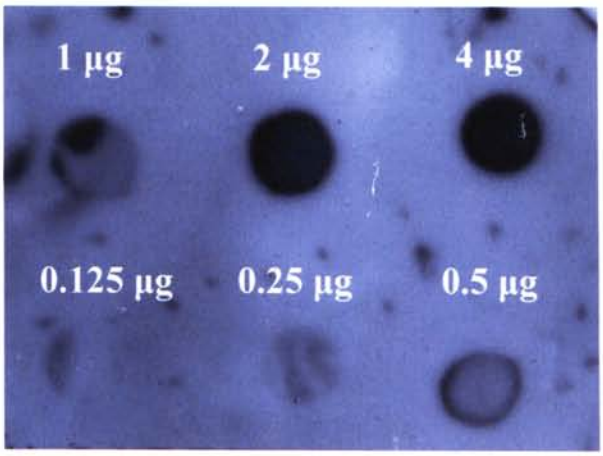


Fig. 3.2. Binding of polyclonal aptamers with cytochrome *c* by dot plot hybridization.

Cytochrome *c* of various amounts were dotted on a PVDF membrane with 1% (w/v) BSA as a non-specific binding blocker. The membrane was then labeled with polyclonal aptamers (1:400) for 1 hour and then incubated with streptavidin conjugated HRP antibody (1:10000) for another hour in binding buffer with 1% (w/v) BSA. ECL assay was performed and signal was exposed on an X-ray film.

3.1.1.3 Monoclonal aptamer

The polyclonal aptamers selected in the previous chapter should have different sequences in the core region. After confirming the polyclonal aptamers that could bind with cytochrome *c*, monoclonal aptamers were produced by cloning them into pBlueScript II vectors.

Briefly, the aptamers were first amplified by PCR with equal concentration of forward and reverse primers to produce double-stranded oligonucleotides. As there were two recognition sites of restriction enzymes *Hind* III and *Eco* RI in the 5' and 3' primer ends of the oligonucleotide respectively, aptamers and pBlueScript II vector were first incubated separately with the two enzymes (1μl each for 1 μg DNA) at 37 °C for 2 hours for the restriction cutting and at 65 °C for 20 minutes to inactivate the enzymes. One percent agarose gel was then used to purify the vector and 12% polyacrylamide gel for the aptamers after reaction. The vectors and aptamers eluted from the gels were then incubated together in a molar ratio of 1:4 with T4 ligase at 16 °C overnight for ligation.

After ligation, the plasmids that contained the aptamers were transformed into competent bacterial cells by heat shock at 43 °C. The transformed competent bacterial cells were then allowed to grow on LB ampicillin agar plates. Since a bacterial colony is grown from a single cell, cells in a single colony should contain a single plasmid with a unique insert and the antibiotic resistance property. Subsequently, bacterial cells were collected from a single colony and culture in LB brooth for overnight at 37 °C. Plasmids were then isolated by alkaline lysis method

buffer. After purifying the plasmid from the bacterial culture of a single colony, monoclonal aptamer was amplified again from the plasmid by PCR (Fig. 3.3).

After these cloning steps, 10 monoclonal aptamers were obtained. However, from the result of the PCR products separated by denaturing gel (Fig. 3.3), one of the clones was discarded (marked with F) as it contained a high and a low molecular weight products after PCR amplification. Only 9 clones were left and were named as cy-1 to cy-9. After amplification, aptamers cy-1 to cy-9 were tested for the binding.

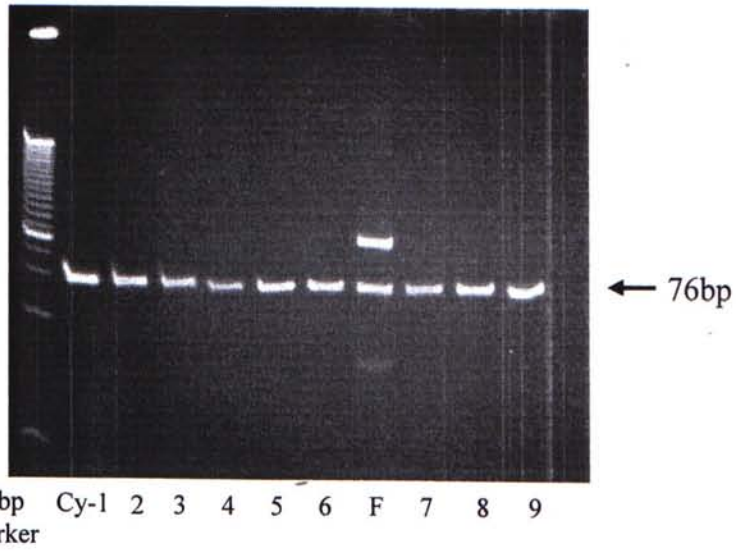


Fig. 3.3. A denaturing gel showing the PCR products amplified from 10 monoclonal plasmids.

One μl of 100-fold diluted plasmids extracted from different colonies were added individually to a PCR reaction mixture (final volume 25 μl). Thirty cycles PCR were performed. The PCR products were then mixed with 6.25 μl urea (7M) and 6.25 μl 6X loading buffer. After heated at 95 °C for 3 minutes, the mixtures and the 25 bp marker were loaded to the wells of 12% polyacrylamide with 7M urea denaturing gel. After separation, DNA bands were visualized by UV with ethidium bromide. The lane marked with 'F' was discarded and the others were named as cy-1 to cy-9.

3.1.2 Binding test of cy-1 to cy-4 to cytochrome *c*

After obtaining the 9 monoclonal aptamers, aptamers cy-1 to cy-4 were tested for the binding to cytochrome *c* by using cytochrome *c* conjugated Sepharose. The cytochrome *c* on the Sepharose were first labeled with aptamers for 15 minutes at room temperature. After washed with binding buffer, the biotin on the aptamers were recognized by streptavidin with HRP conjugated. OPD (0.8 mg/ml) in substrate buffer with 0.03% H_2O_2 was then added as the substrates for HRP, and the reaction was stopped by 0.1M sulfuric acid. Yellowish products were measured at OD 492 nm.

The results of the binding test are shown in Fig. 3.4. From the graph, aptamers cy-1 to cy-4 were found to bind to cytochrome *c* when compared to the negative one which had no aptamer added. Among the four aptamers tested, cy-3 was shown to have the highest affinity to the cytochrome *c*. And thus, cy-3 was chosen for further study.

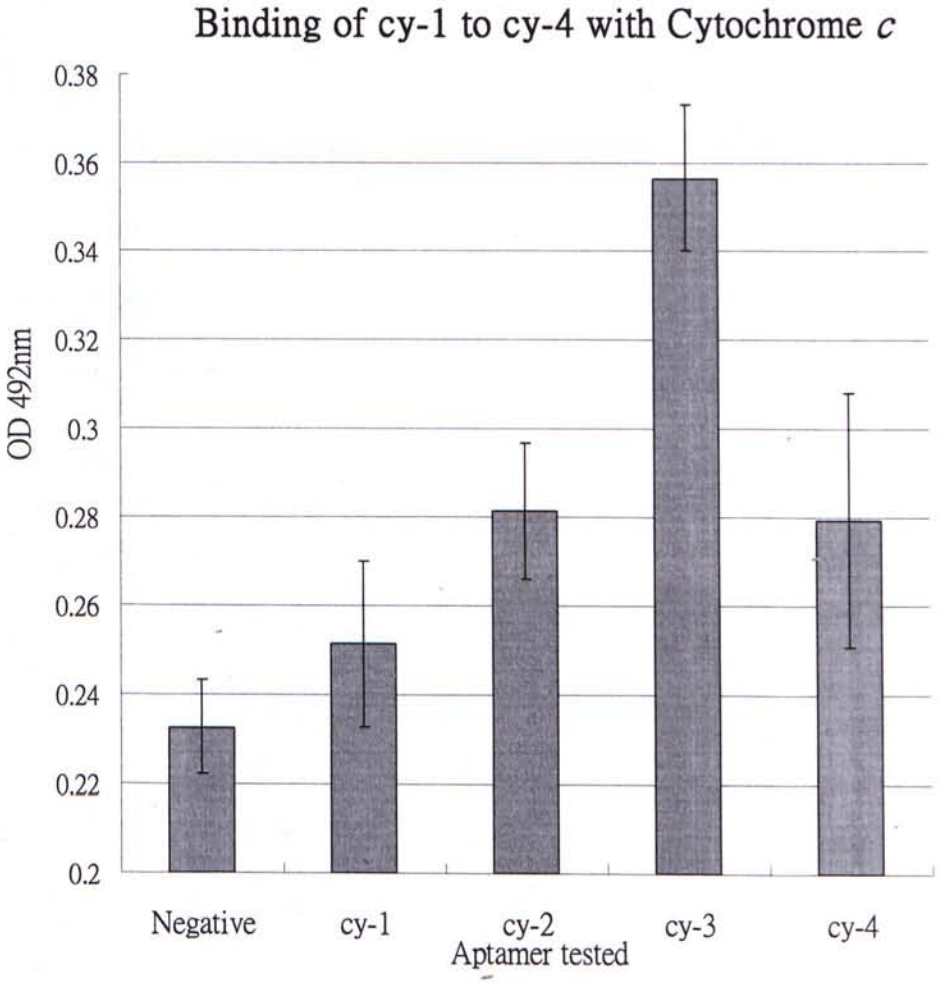


Fig. 3.4. Binding of aptamers cy-1 to cy-4 with cytochrome *c* conjugated on Sepharose.

Cytochrome *c* conjugated Sepharose (20 µl) was incubated with 50 µl PCR products of cy-1 to cy-4 amplified from plasmid in 400 µl binding buffer or binding buffer only (negative) (total volume) for 15 minutes at room temperature. After washing, streptavidin HRP (1:10000) was added and incubated for another 15 minutes. After washing, OPD substrate was then added and absorbance was measured at 492 nm. Results are mean ± SD of 3 determinations.

3.1.3 Binding of cy-3 to the cytochrome *c* dotted on PVDF membrane

With the monoclonal aptamer cy-3, Western blot analysis with cytochrome *c* of various concentrations was performed again. Cy-3 was amplified from purified plasmid by PCR with excess biotinylated 5' forward primer. PCR product was used to label the cytochrome *c* dotted on PVDF membrane.

Results below show that the cy-3 could label cytochrome *c* in a dose dependent manner (Fig. 3.5). In this Western blot, 0.25 μg of cytochrome *c* seemed to be the detection limit.

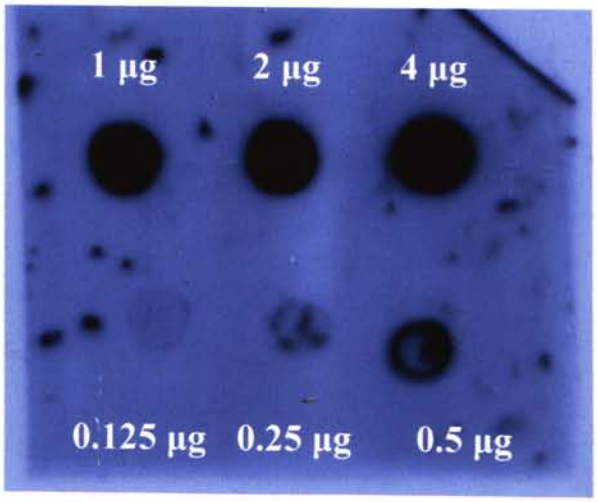


Fig. 3.5. Binding of cy-3 with cytochrome *c* by dot plot.
Cytochrome *c* of various amounts was dotted on a PVDF membrane with 1% (w/v) BSA as a non-specific binding blocker. The membrane was then labeled with cy-3 (1:400) for 1 hour and then incubated with streptavidin conjugated HRP (1:10000) for another hour in binding buffer with 1% (w/v) BSA. ECL assay was performed and signal was exposed on an X-ray film.

3.1.4 Test the binding of cy-3 with cytochrome *c* by ELISA

In this assay, we were trying to study the binding of cy-3 to cytochrome c by ELISA. Cytochrome *c* was first coated on a 96 wells plate in 2-fold dilutions and non-specific blocking was done by 1% (w/v) BSA. Cy-3 (1 nmol/ml, 100 μl) in 1% (w/v) BSA binding buffer was added to each well after heating at 95 °C for 3 minutes. After incubating for 1 hour, the wells were washed with binding buffer for 5 times. One hundred μl streptavidin HRP (1:10000) in 1% (w/v) BSA binding buffer was added to each well and incubated for another hour. OPD as the substrate of HRP was added to each well and incubated for 30 minutes at 37 °C. Yellowish colour product was then measured at OD 492 nm.

The results are shown in Fig. 3.6. From the graph, cy-3 was found to bind cytochrome *c* in a dose-dependent manner. The amount of yellowish product produced increased with the amount of cytochrome *c* until it leveled off when the amount of cytochrome *c* was higher than 4 μg.

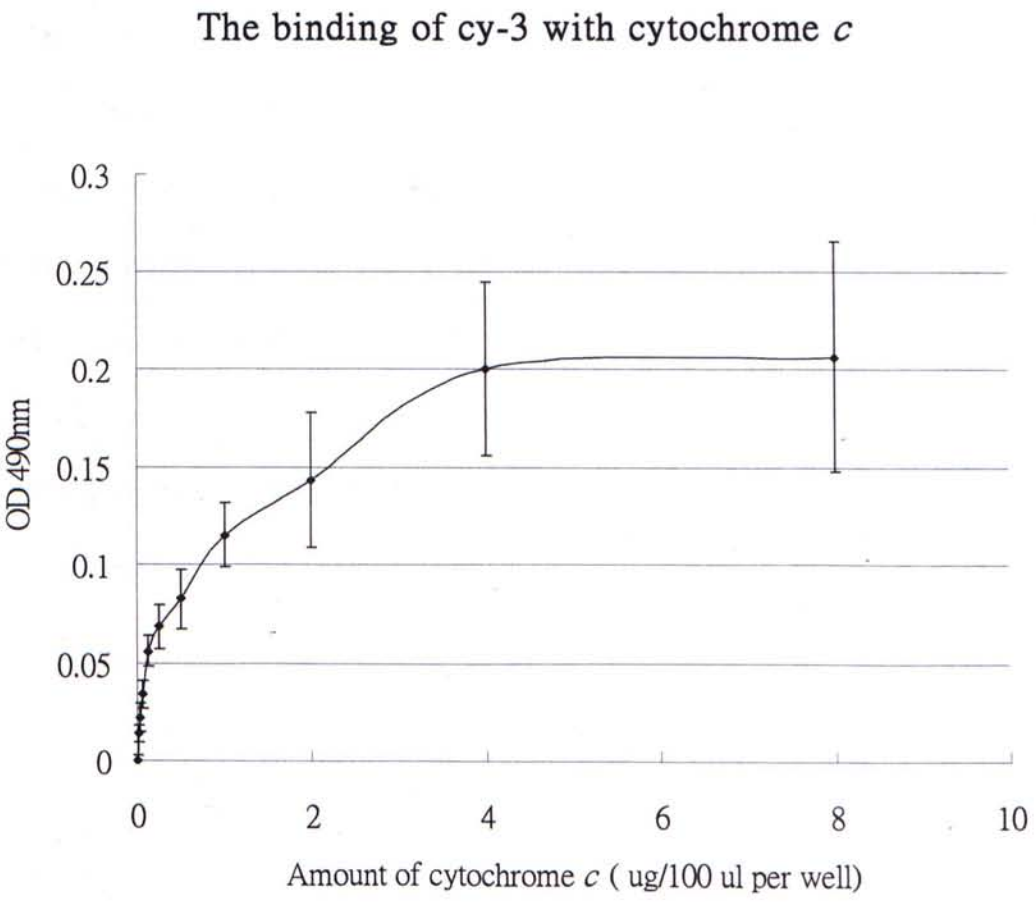


Fig. 3.6. Binding of cy-3 with cytochrome *c* in ELISA assay.
Cytochrome *c* was coated on 96 well-plate in 2-fold dilutions started at concentration 8 μg /100 μl per well. After labeling with 100 pmol cy-3 and streptavidin, OPD substrate was added. OD at 490 nm was then measured which is directly proportional to the amount of yellowish product produced. Results are mean ± SD of 3 determinations.

3.1.5 Competitive binding between monoclonal aptamer cy-3 and anti-cytochrome *c* antibody

To test whether cy-3 and the anti-cytochrome *c* antibody bind to the same epitope of cytochrome *c*, a competitive ELISA was performed. In this assay, cytochrome *c* was first blocked with a decreasing amount of monoclonal aptamer cy-3. After washing, the blocked cytochrome *c* was labeled with anti-cytochrome *c* and anti-mouse HRP conjugated antibody. From the results, it was found that no inhibition was observed suggesting that monoclonal aptamer cy-3 could not block the binding between anti-cytochrome *c* antibody and cytochrome *c*. This may due to the fact that monoclonal aptamer and the antibody recognized different epitopes of cytochrome *c*.

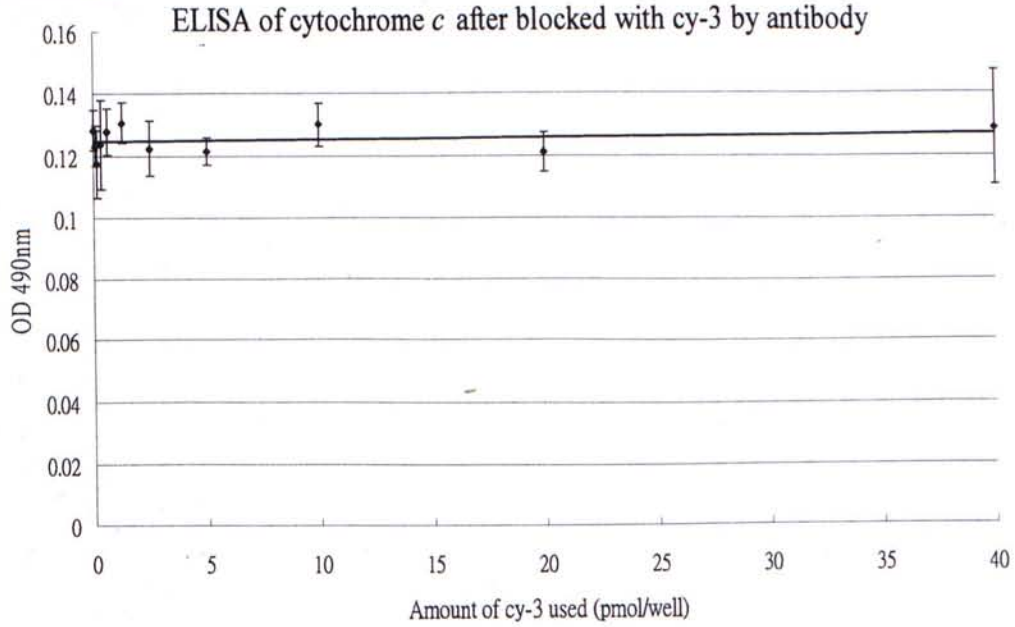


Fig. 3.7. Competitive ELISA of cytochrome *c* between aptamer cy-3 and anti-cytochrome *c* antibody

Cytochrome *c* was coated on a 96 wells plate at a concentration of 1 μg/ 100 μl per well. Different amounts of gel-purified cy-3 were added and then incubated for 1 hour. After washing, cytochrome *c* were quantified by anti-cytochrome *c* antibody (1:10000) and anti-mouse antibody with HRP (1:10000) in 0.1% (w/v) BSA binding buffer. After washing, OPD substrate was added. OD at 490 nm was then measured. Results are mean ± SD of 3 determinations.

3.1.6 Western blotting of pure cytochrome *c* by cy-3

During the course of studying of apoptotic proteins, we found that commercially available cytochrome *c* contains a number of dimers, trimers or polymers of cytochrome *c* after SDS-PAGE. To examine whether cy-3 reacts with the polymers, cy-3 was tested for the binding of cytochrome *c* after SDS-PAGE.

As shown in Fig. 3.8, cy-3 could label the cytochrome *c* only at the position of 15 kDa while anti-cytochrome *c* antibody labeled some high molecular weight proteins. The high molecular weight proteins may be the dimers, trimers or tetramers of cytochrome *c*. However, the sensitivity of the cy-3 against cytochrome *c* was lowered than that of antibody as the detection limit of cy-3 was found to be 0.5 μg at the condition we used.

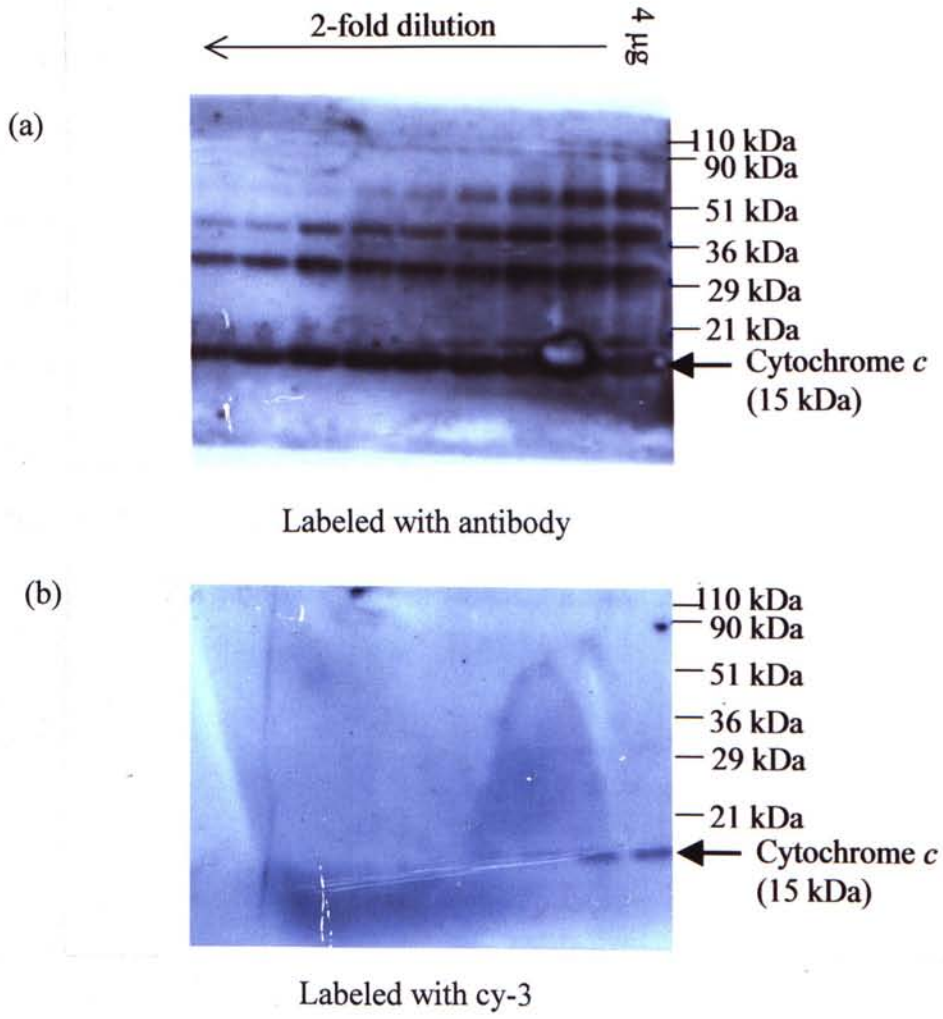


Fig. 3.8. Binding of cy-3 with cytochrome *c* after SDS-PAGE.

Pure cytochrome *c* was loaded to the lanes of a 12% SDS-PAGE from right to left with the amount as indicated. After electrophoresis, the proteins were then transferred to PVDF membranes and non-specific blocking was made by 1% (w/v) BSA. The membranes were then labeled with (a) anti-cytochrome *c* antibody (1:2000) or (b) cy-3 (1:400) in 1% (w/v) BSA TBS-T or binding buffer for 1 hour respectively and then labeled with (a) anti-mouse HRP conjugated antibody (1:1000) or (b) streptavidin HRP (1:10000) respectively for another hour. ECL assay was done and signal was generated on an X-ray film.

3.1.7 Western blotting of pure cytochrome c from different species

The cytochrome *c* as the target of the selection in the pervious chapters was extracted from horse. In this chapter, cytochrome *c* from different species (chicken, horse, rat, tuna and yeast) were separated by 15% SDS-PAGE and transferred to PVDF membranes. They were then labeled with anti-cytochrome *c* antibody and monoclonal aptamer cy-3.

From the results (Fig. 3.9), it was found that cy-3 could label cytochrome *c* from all the species tested, but antibody could not label the cytochrome *c* from yeast. This suggests that cy-3 bind to a universal common site on cytochrome *c*. Moreover, proteins bands with a higher or lower molecular size of 15 kDa were observed in the blotting with antibody. No such observation was found with aptamers.

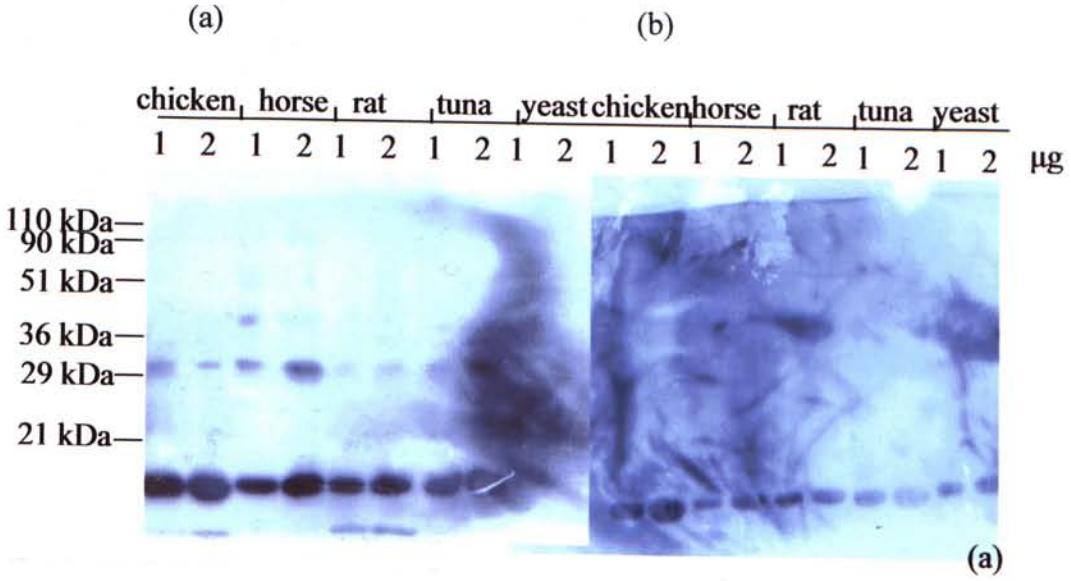


Fig. 3.9. Different species of cytochrome *c* labeled with anti-cytochrome *c* antibody and monoclonal cy-3.

Cytochrome *c* of chicken, horse, rat, tuna and yeast (1 and 2 µg) were separated by 15% SDS-PAGE. The separated proteins were then transferred to PVDF membranes and non-specific binding was blocking by 1% (w/v) BSA. The membranes were then labeled with (a) anti-cytochrome *c* antibody (1:2000) or (b) cy-3 PCR product (1:400) in 1% (w/v) BSA TBS-T or binding buffer respectively for 1 hour. Subsequently, membranes were labeled with (a) anti-mouse HRP conjugated antibody (1:1000) or (b) streptavidin HRP (1:10000) respectively for another hour. After washing, ECL assay was done and signal was exposed on an X-ray film.

3.1.8 Cell lysate SDS-PAGE labeled with cy-3

After testing the cy-3 for pure cytochrome *c*, we then examined whether cy-3 could bind to the cytochrome *c* in a mixture of cell lysate by Western blotting. By using lysis buffer with digitonin (400 μg/ 4 x 10^6 cells), total cell proteins including those in mitochondria were extracted. The total cell lysate was then separated by SDS-PAGE and transferred to a PVDF membrane. After blocking with 1% (w/v) BSA, the membrane was labeled with cy-3 or anti-cytochrome *c* antibody.

The results shown in Fig. 3.10 indicate that cy-3 labeled the cytochrome *c* as well as other cytosolic proteins. In the case with antibody, cytosolic proteins other than cytochrome *c* were also observed.

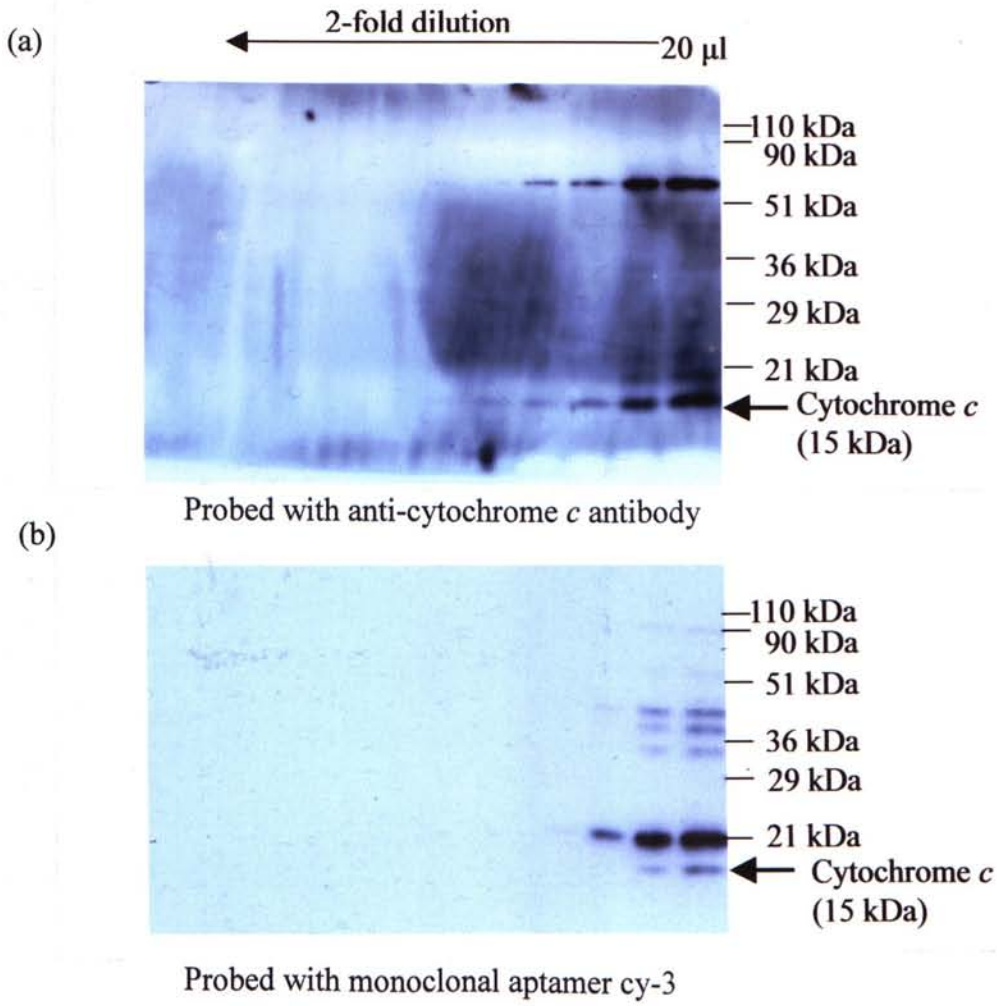


Fig. 3.10. Binding of cy-3 and anti-cytochrome *c* antibody with total cell lysate.

L929 total cell lysate was extracted with lysis buffer containing a high dose of digitonin (400 µg/ 4 x 10^6 cells). Cellular proteins were then separated by 12% SDS-PAGE. Started from the right, 20 µl of the total cell lysate was loaded to the wells with a 2-fold dilutions. The separated proteins were then transferred to PVDF membranes and non-specific binding was blocked by 1% (w/v) BSA. The membranes were then labeled with (a) anti-cytochrome *c* antibody (1:2000) or (b) cy-3 PCR product (1:400) in 1% BSA (w/v) TBS-T or binding buffer respectively for 1 hour. Subsequently, membranes were labeled with (a) anti-mouse HRP conjugated antibody (1:1000) or (b) streptavidin HRP (1:10000) respectively for another hour. After washing, ECL assay was done and signal was exposed on an X-ray film.

3.1.9 Cell lysate labeled with cy-1 to cy-9 after SDS-PAGE

From the result of Fig. 3.10, it was found that cy-3 labeled other cytosolic proteins. This may be due to the fact that the sequence of the cy-3 produced a unique 3 dimensional structure that bound the cytosolic proteins with a common core structure. Therefore, cy-3 may not be a specific clone of aptamer just for cytochrome *c*.

In this connection, other 8 clones, cy-1, cy-2, cy-4 to cy-9, were amplified from plasmid by using PCR and their ability to bind to cytochrome *c* from cell lysate was investigated.

Results in Fig. 3.11 show that all the monoclonal aptamers bound cytochrome *c* and other cytosolic proteins with a similar pattern.

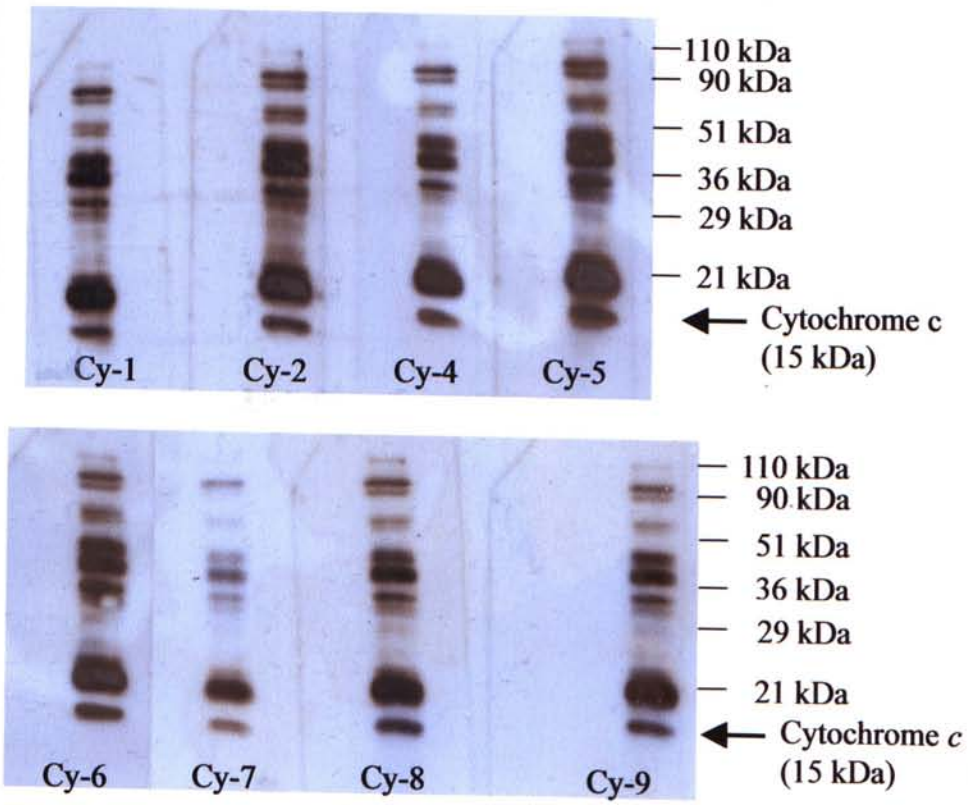


Fig. 3.11. Binding of cy-1, cy-2 and cy-4 to cy-9 with cell lysate after SDS-PAGE.

L929 total cell lysate was extracted with lysis buffer containing a high dose of digitonin (400 μg/ 4 x 10^6 cells). Cellular proteins were then separated by 12% SDS-PAGE and transferred to PVDF membranes. Non-specific binding was blocked by using 1% (w/v) BSA. The membranes were then labeled with cy-1, cy-2 and cy-4 to cy-9 PCR products (1:400) as indicated in 1% (w/v) BSA binding buffer for 1 hour. Subsequently, membranes were labeled with streptavidin HRP (1:10000) for another hour. After washing, ECL assay was done and signal was exposed on an X-ray film.

3.2 Selection of cytochrome *c*-specific aptamer with counter selection against cytosolic protein

The results above (Fig. 3.10 and Fig. 3.11) show that the aptamers selected bound to cytochrome *c* and other cytosolic proteins. This may be due to a possibility that the aptamers selected (cy-1 to cy-9) might fold into 3-dimensional structures that could recognize cytosolic proteins.

To eliminate these unwanted bindings, the pool of oligonucleotides was first counter selected against cytosolic proteins before the selection with cytochrome *c*. Those aptamers bound to cytosolic proteins would be discarded.

To increase our success rate, we employed three different methods for the counter selection against the cytosolic proteins.

3.2.1 Selection of aptamer against cytochrome *c* with counter selection against cytosolic cell lysate

In the first method, the cytosolic cell lysate was extracted from growing L929 with lysis buffer containing a low dose of digitonin (12.5 μg/ 4 x 10^6 cells). By using this dosage of digitonin, only the plasma membrane was permeabilized but not the mitochondrial membrane. Thus, proteins in mitochondria including cytochrome *c* will not be extracted. After extraction, cytochrome *c* free cytosolic proteins were dotted on a PVDF membrane for counter selection.

In this preparation, 100 pmol of the oligonucleotides in the library were used. It first performed a cycle of counter selection against BSA on PVDF membranes. Those oligonucleotides that did not interact with BSA membrane were collected. In the second round selection, oligonucleotides from the first round reaction were incubated with cytochrome *c* on PVDF membrane. After washing and elution, oligonucleotides reacted with cytochrome *c* were amplified by PCR. The products were subject to another round of counter selection against cytosolic cell lysate (which is extracted with lysis buffer containing a low dose of digitonin as mentioned before) saturated on PVDF membrane for about 6 hours followed by a selection against cytochrome *c* coated on PVDF membrane.

The aptamers selected were then amplified again and biotinylated. Western blot was used to test the binding of the aptamer to the total cell lysate and the results are shown in Fig. 3.12.

From the results, it can be found that the aptamer selected could bind both the cytochrome *c* and cytosolic proteins even after counter selection with the cytochrome *c* free cytosolic proteins. Also, the binding pattern was similar to those observed in Fig. 3.11.

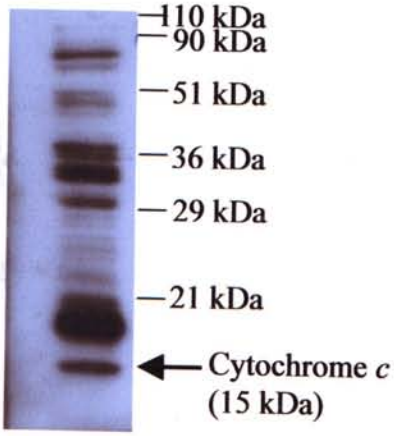


Fig. 3.12. Effect of the counter-selection with cytosolic proteins on the selection of cytochrome *c* specific aptamer.

Total cell lysate of L929 was extracted with lysis buffer containing a high dose of digitonin (400 μg/ 4 x 10^6 cells). Cellular proteins were separated by 15% SDS-PAGE and transferred to PVDF membranes. Non-specific binding was blocked by using 1% (w/v) BSA. The membranes was then labeled with the aptamer (selected after counter selection with cytochrome *c* free cytosolic proteins) for 1 hour in 1% (w/v) BSA binding buffer and then labeled with streptavidin HRP (1:10000) for another hour. ECL assay was done and signal was exposed on an X-ray film.

3.2.2 Selection of aptamer against cytochrome *c* by fixed cell followed by cytochrome *c* elution

In the second counter selection, process was started by using 100 pmol oligonucleotides from the pool with 40 random nucleotides. To eliminate those oligonucleotides that could bind the blocking agent BSA, the pool was first counter selected against BSA for 1 hour. The oligonucleotides remained in the binding buffer was transferred and incubated with fixed cells for 2 hours. After washing with binding buffer, cytochrome *c* specific aptamers were then eluted by 400 μl 100 μg/ml cytochrome *c* in binding buffer with decreasing time intervals from 1 hour, 45 minutes, 30 minutes or 15 minutes in the final round of selection for the high affinity aptamers.

Those aptamers selected were amplified by PCR with biotinylated 5' forward primer. The biotinylated aptamers were then used to label the cell lysate after SDS-PAGE (Fig. 3.13). Unfortunately, aptamers were found to bind to cytochrome *c* and cytosolic proteins. Also, it seems likely that the affinity between the aptamers and cytochrome *c* was not strong. Yet, the binding pattern was different from Fig. 3.11 and 3.12.

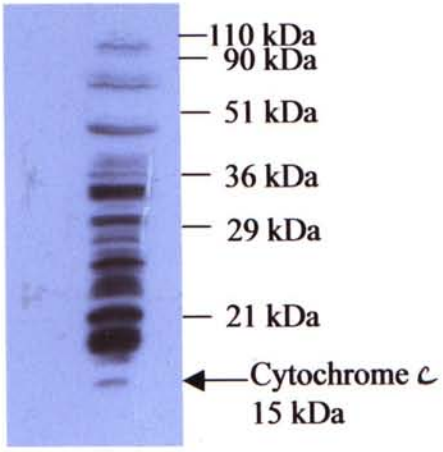


Fig. 3.13. Western analysis of the binding of cell lysate to aptamers selected by fixed cell followed by cytochrome *c* elution.

L929 total cell lysate was extracted with lysis buffer containing a high dose of digitonin (400 μg/ 4 x 10^6 cells). Cellular proteins were then separated by 15% SDS-PAGE and transferred to a PVDF membrane. Non-specific binding was blocked by using 1% (w/v) BSA. The membranes were then labeled in 1% (w/v) BSA binding buffer for 1 hour with the aptamers selected by fixed cell and eluted by cytochrome *c*. Subsequently, membranes were labeled with streptavidin HRP (1:10000) for another hour. After washing, ECL assay was done and signal was exposed on an X-ray film.

3.2.3 Selection of aptamer from cytochrome *c* band

In the Western blot analysis, the aptamer selected was found to bind to both the cytosolic protein and cytochrome *c* on PVDF membrane (Fig. 3.12). To have cytochrome *c* specific aptamer, aptamers on the band of cytochrome *c* should be highly specific to the cytochrome *c*. With this rationale in our mind, we cut the band of cytochrome *c* out of the PVDF membrane labeled with the aptamers selected with counter selection against cytosolic cell lysate (section 3.2.1) and eluted the aptamers by elution buffer. After amplification by PCR and biotinylation, the aptamer eluted were tested for protein binding again with Western blot analysis (Fig. 3.14).

From the results (Fig. 3.14), it can be seen that the aptamer eluted from cytochrome *c* band could bind to cytochrome *c* and cytosolic proteins. Interestingly, the binding pattern was similar to the one observed in Figs. 3.11 and 3.12.

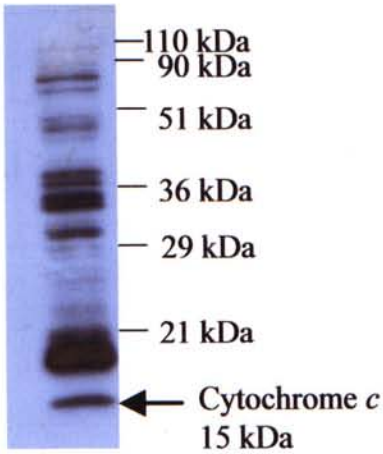


Fig. 3.14. Western analysis of the binding of aptamers from the cytochrome *c* band to cytosolic proteins.

Total cell lysate of L929 was extracted with lysis buffer containing a high dose of digitonin (400 μg/ 4 x 10^6 cells). Cellular proteins were separated by 15% SDS-PAGE and transferred to a PVDF membrane. Non-specific binding was blocked by using 1% (w/v) BSA. The membranes was then labeled with the aptamer eluted from the band of cytochrome *c* for 1 hour in 1% (w/v) BSA binding buffer and then labeled with streptavidin HRP (1:10000) for another hour. The ECL assay was done and signal was exposed on an X-ray film.

3.3 Primers Testing

3.3.1 Cell lysate labeled with primers after SDS-PAGE

All the clones (cy-1 to cy-9) tested were found to label cytosolic proteins with a similar pattern (Fig. 3.11). And even the aptamers eluted from the band of cytochrome *c* could bind to other cytosolic protein in a similar way (Fig. 3.12 and 3.14). This may be due to the presence of a common item in the labeling solution that leads to the non-specific binding of cytosolic protein. In our study, all the tested clones were developed by PCR reactions in which excess amount of biotinylated forward 5' primers were added. This is necessary because without the biotinylated forward 5' primer, no signal will be given since it contains biotin for the recognition of streptavidin HRP and it should be in excess to make sure that aptamers so produced were the single sense strand. In light of this, biotinylated forward 5' primers were used to examine whether the forward 5' primers label the cell lysate on membrane.

Results from Fig. 3.15 show that biotinylated forward 5' primers could label cytosolic proteins but not cytochrome *c*. Also, the binding pattern was different from the previous ones. These observations suggest that the non-specific binding with the cytosolic proteins other than cytochrome *c* in the results of the previous chapters might be due to the presence of excess primers in the reaction mixture.

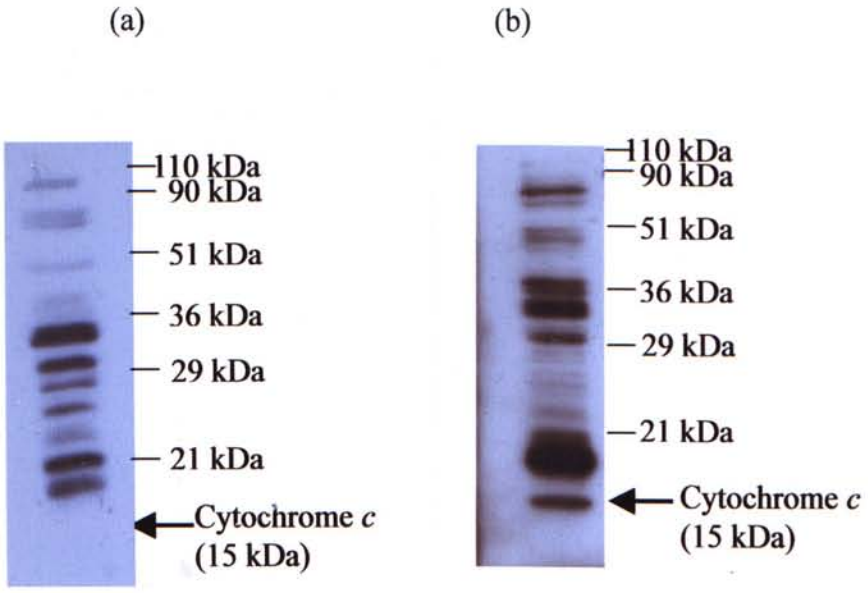


Fig. 3.15. Western analysis of the forward 5' primers with cell lysate.
L929 total cell lysate was extracted with lysis buffer containing a high dose of digitonin (400 μg/ 4 x 10^6 cells). Cellular proteins were then separated by 15% SDS-PAGE and transferred to PVDF membranes. Non-specific binding was blocked by using 1% (w/v) BSA. The membranes were then labeled with 100 pmol biotinylated 5' primer (a) or aptamer selected with counter selection against cytochrome *c* free cell lysate (b) in 1% (w/v) BSA binding buffer for 1 hour and then labeled with streptavidin HRP (1:10000) for another hour. ECL assay was done and signal was exposed on an X-ray film.

3.3.2 Cell lysate labeled with cy-3 without primer

Since the biotinylated 5' forward primer was found to bind cytosolic proteins (Fig. 3.15), they were eliminated from the aptamers before labeling with the total cell lysate. The PCR products of cy-3 were purified by using 12% polyacrylamide with 7 M urea denaturing gel. The cy-3 were then eluted from the gel and used to label cell lysate separated by SDS-PAGE and the Western analysis was shown in Fig. 3.16.

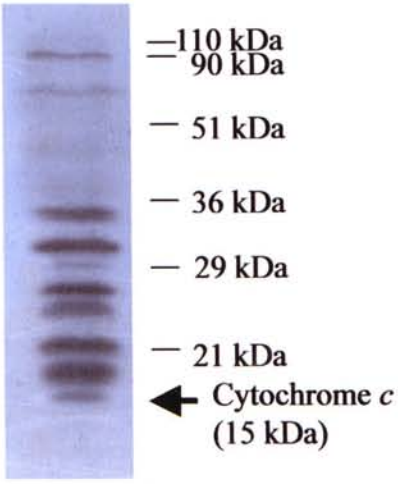


Fig. 3.16. Western analysis of purified cy-3 without the contamination of forward 5' primers with cell lysate.

L929 total cell lysate was extracted with lysis buffer containing a high dose of digitonin (400 μg/ 4 x 10^6 cells). The cellular proteins were then separated by 15% SDS-PAGE and transferred to a PVDF membrane and non-specific binding was blocked by using 1% (w/v) BSA. The membranes were then labeled with purified cy-3 (without biotinylated 5'primer) in 1% (w/v) BSA binding buffer for 1 hour and then labeled with streptavidin HRP (1:10000) for another hour. ECL assay was done and signal was exposed on an X-ray film.

From the results (Fig. 3.16), it can be seen that cy-3 after the removal of the excess 5' primers still bound to other cytosolic proteins non-specifically in addition to cytochrome *c*. However, the binding pattern was different from the one shown in Fig. 3.11.

3.3.3 Test the effect of sense oligonucleotide

Next, we tried to examine whether the non-sense oligonucleotides produced during PCR contributed to the non-specific binding in the Western blot analysis. After the PCR and purification by denaturing gel, the biotinylated aptamers were captured by using streptavidin paramagnetic beads after heated at 95 °C. Under this condition, only the sense strands were captured and they were then eluted and used for labeling.

From the results, the biotinylated sense aptamer strands showed non-specific binding also in the absence of the antisense-oligonucleotides and primers.

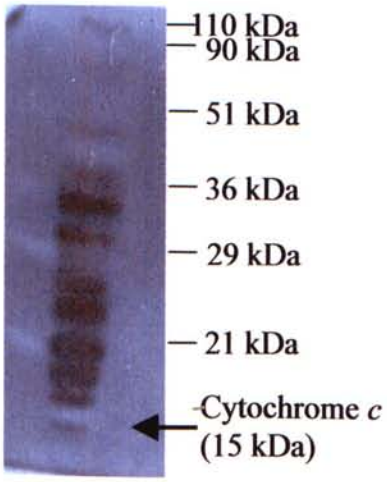


Fig. 3.17. Western analysis of purified cy-3 without the contamination of non-sense oligonucleotides with cell lysate.

L929 total cell lysate was extracted with lysis buffer containing a high dose of digitonin (400 μg/ 4 x 10^6 cells). The cellular proteins were then separated by 15% SDS-PAGE and transferred to a PVDF membrane and non-specific binding was blocked by using 1% (w/v) BSA. The membranes were then labeled with purified cy-3 (without biotinylated 5'primer and the antisense oligonucleotide) in 1% (w/v) BSA binding buffer for 1 hour and then labeled with streptavidin HRP (1:10000) for another hour. ECL assay was done and signal was exposed on an X-ray film.

3.3.4 Sequence of monoclonal aptamer

With the results from Fig. 3.15 and 3.16, it is possible that the primer sequence at the 5' end of the cy-3 aptamers attached to the cytosolic proteins. To investigate this possibility, we tried to remove the primer sequences from aptamers cy-1 to cy-3. Yet, after the elimination of the primer sequence from the aptamers, the aptamers could not be produced by PCR anymore. The aptamers could only be synthesized by oligonucleotide synthesizer according to the sequence. Thus, aptamers cy-1 to cy-3 were sequenced by an automated DNA sequencer. Their flanked primer sequences and core sequences are shown in Fig. 3.18a. The predicted secondary structures of the aptamers with or without flanked primers are also illustrated in Fig. 3.18. As shown in Fig. 3.18 b and c, the secondary structures of cy-1 to cy-3 are different.

(a)

5' ATCGAT**AAGCTT**CCAGAG-
Conserved 5' region for primer binding
Cy-1: GAACTAGAGTTGCATCTCTCGCCGGCTTACGGCTCGAACC

Cy-2: GTAGGGTGGTGAGCTCGGGGTTATCTCCCTAGGGCCATTAC

Cy-3: CCGTGTCTGGGGCCGACCGGCGCATTGGGTACGTTGTTGC

-CGTA***GAATTC***CTGCAGCC 3'
Conserved 3' region for primer binding

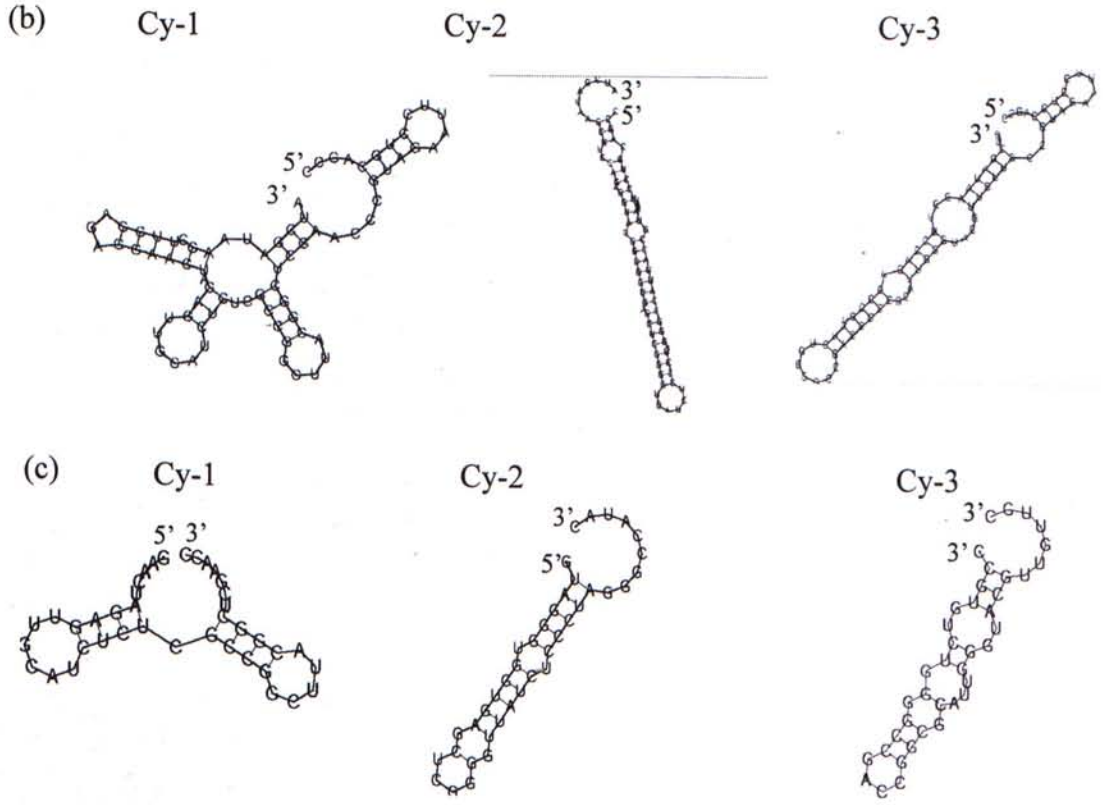


Fig. 3.18. The sequences and the secondary structures of aptamer cy-1 to cy-3.

(a) The flanked primer sequence and the core sequence. Bold letters show the cutting site for Hind III enzyme. Bold italic letters show the cutting site for Eco RI enzyme.

With the nucleotide sequences, the predicted secondary structures of the aptamer cy-1 to cy-3 with (b) or without (c) flanked primers were predicted by using Vienna RNA Secondary Structure Prediction and Comparison Programme (http://rna.tbi.univie.ac.at/~ivo/RNA).

3.3.5 Cell lysate labeled with aptamers without primer ends

The core sequence of aptamer cy-3 was synthesized according to the result of sequencing with a biotin conjugated at 5' end. The synthesized aptamer was called BS-cy3. BS-cy3 was then allowed to react with cell lysate and results are shown in Fig. 3.19. Unfortunately, the core sequence of the aptamer cy-3 which did not have the primer sequences also labeled cytosolic proteins and cytochrome *c*. However, the binding pattern was unique and more cytosolic proteins were labeled.

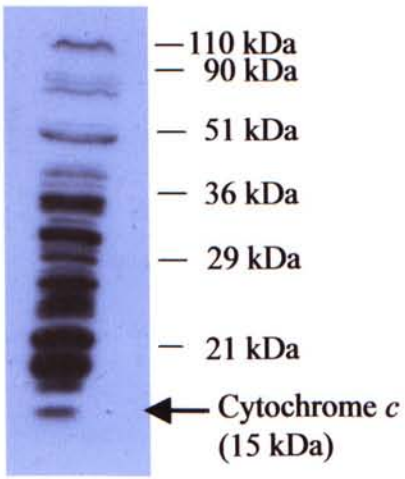


Fig. 3.19. Western analysis of BS-cy3 with cell lysate after SDS-PAGE.
L929 total cell lysate was extracted with lysis buffer containing a high dose of digitonin (400 μg/ 4 x 10^6 cells). Cellular proteins were then separated by 15% SDS-PAGE and transferred to a PVDF membrane. Non-specific binding was blocked by using 1% (w/v) BSA. The membranes were then labeled with 100 pmol BS-cy3 (the core sequence of the aptamer cy-3, without the 5' forward and 3' reverse primers sequences) in 1% (w/v) BSA binding buffer for 1 hour and then labeled with streptavidin HRP (1:10000) for another hour. ECL assay was done and signal was exposed on an X-ray film.

3.3.6 Test of the aptamers after mutations

In this part, we introduced point mutations in the monoclonal aptamer cy-3 according to the result of sequencing. By the prediction of the software, the secondary structures of the mutants were different from that of cy-3. Cy-3 and its mutants including the flanked ends were synthesized with biotin conjugated at 5' end and tested for the binding to cytochrome *c* by Western blotting.

With the mutation, the secondary structures of the mutants were found to be different from the original aptamer (Fig. 3.20) After testing for labeling, our results showed that cy-3-3 could bind to cytochrome *c* with a higher affinity (in terms of intensity) after mutation while cy-3-2 showed a lower affinity to the cytochrome *c* (Fig. 3.21). Cy-3-2 also showed a non-specific binding as a small band at the size of <15 kDa next to the band of cytochrome *c*.

(a)

Cy-3

5' ATCGATAAGCTTCCAGAG-
CCGTGTCTGGGGCCGACCGGCGCATTGGGTACGTTGTTGC
-CGTAGAATTCCTGCAGCC 3'

Cy-3-2

5' ATCGATAAGCTTCCAGAG-
CCGTGTCTGGGGCCGACCAACGCATTGGGTACGTTGTTGC
-CGTAGAATTCCTGCAGCC 3'

Cy-3-3

5'ATCGATAAGCTTCCAGAG-
CCGTGTCTGGGGCCGACCGGCGCATTTGATACGTTGTTGC
-CGTAGAATTCCTGCAGCC 3'

(b)

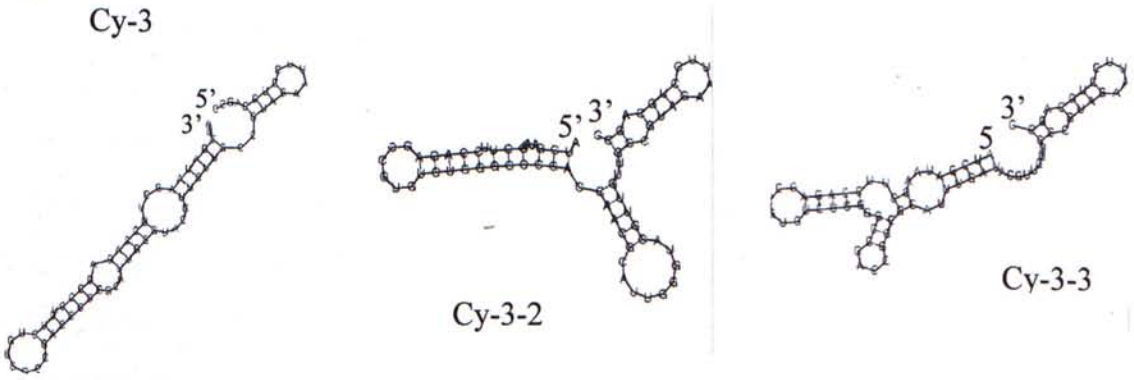


Fig. 3.20. The sequences and the secondary structures of aptamers after mutation.

(a): The sequences of the original aptamers cy-3 and its mutants (cy-3-2 and cy-3-3). The nucleotides underlined were the points of mutation. (b): With the prediction by using Vienna RNA Secondary Structure Prediction and Comparison Programme, the secondary structures of the aptamers before and after mutation were shown.

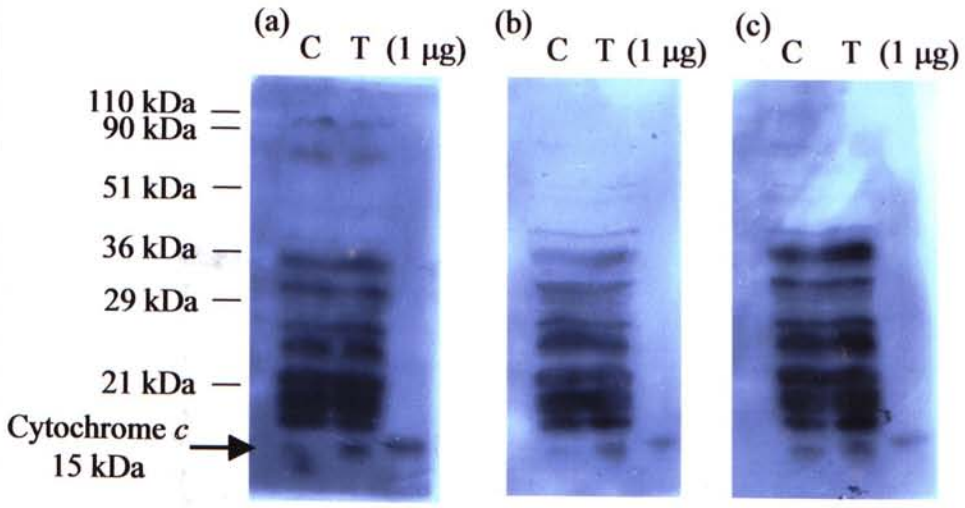


Fig. 3.21. Labeling of cell lysate with mutants of cy-3.

L929 were seeded in a 6 wells plate (1 x 10^6 cells per well) overnight. The cells were then incubated with medium alone (C) or TNF-α (50 ng/ml) (T) for 6 hours at 37 °C, 5% CO_2. After treatment, the cells were washed with PBS and total cell lysate were extracted with lysis buffer containing a high dose of digitonin (400 μg/ 4 x 10^6 cells). Cellular proteins and 1 μg cytochrome *c* (1 μg) were then separated by 15% SDS-PAGE. The separated proteins were then transferred to PVDF membranes. Non-specific binding was blocked by 1% (w/v) BSA. The membranes were then labeled with 100 pmol (a) cy-3, (b) cy-3-2 or (c) cy-3-3 in 1% (w/v) BSA binding buffer for 1 hour and then labeled with streptavidin HRP (1:10000) for another hour. ECL assay was done and signal was exposed on X-ray films.

3.3.7 Test for other biotinylated primer

In the literature, single protein band was developed with a monoclonal aptamer against cellular proteins ERK2 in the Western blot analysis (Bianchini *et al.*, 2001). This suggests that their primer set might be a good start for aptamer development. With this thought in our mind, same primers with biotin conjugated were synthesized and their binding to cell lysate was examined. The sequences of the primers used in this experiment were:

ERK2-FOR: TCCATTCACAGACCTTTTGAGC

ERK2-REV: GACACACGCCTCTATCATATCA

Labeling the cell lysate with this pair of primers in the condition we used was performed, results were different from the work done by Bianchini *et al.* (2001). Our results in Fig. 3.22 show that the primers also bound the cytosolic proteins. Interestingly, the binding pattern of ERK2-FOR and ERK2-REV was more or less the same (Fig. 3.22). However, with the labeling condition used by Bianchini *et al.*, that is blocked the PVDF membrane and labeling with 5% (w/v) milk, his primer pair did not show any non-specific binding to the cytosolic proteins.

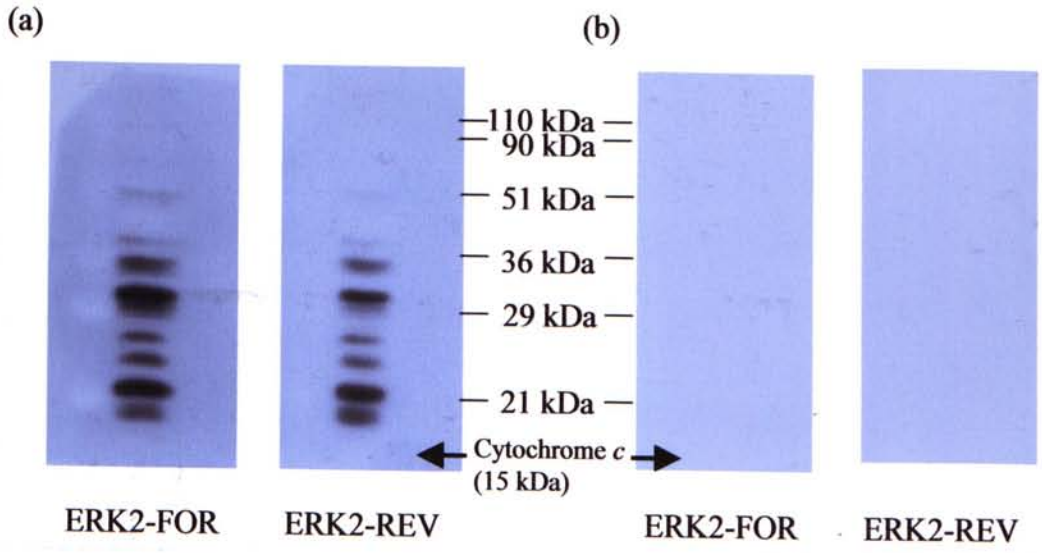


Fig. 3.22. Western analysis of the ERK2-Forward and ERK2-Reverse primers with cell lysate.

L929 total cell lysate was extracted with lysis buffer containing a high dose of digitonin (400 μg/ 4 x 10^6 cells). Cellular proteins were then separated by 15% SDS-PAGE and transferred to PVDF membranes. Non-specific binding was blocked by using (a) 1% (w/v) BSA or (b) 5% (w/v) milk. The membranes were then labeled with the forward or reverse primers (100 pmol) in (a) 1% (w/v) BSA or (b) 5% (w/v) milk in binding buffer for 1 hour and then labeled with streptavidin HRP (1:10000) for another hour. ECL assay was done and signal was exposed on X-ray films.

3.4 Elimination of non-specific binding

3.4.1 Different types of cell lysate

To test if other cell lysate can be labeled non-specifically with the biotinylated primers, cell lysates of different cell lines including L929, HL-60 and HepG2 cells were collected. After Western blotting, the cell lysates were labeled with biotinylated primer (the pair used in our own selection) (Fig. 3.23).

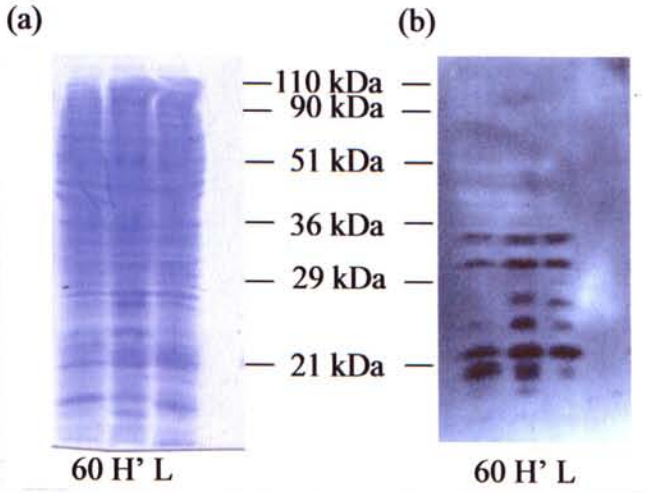


Fig. 3.23. Western analysis primer with cytosolic proteins from different cell lines.

Total cell lysate of L929 (L), HepG2 (H') and HL 60 (60) were extracted with lysis buffer containing a high dose of digitonin (400 μg/ 4 x 10^6 cells). Cellular proteins were separated by 15% SDS-PAGE and transferred to PVDF membranes. One of the membranes was stained by coomassie blue (a) and the other one was incubated with 1% (w/v) BSA for non-specific blocking (b). The membranes blocked were then labeled with biotinylated 5' forward primer in 1% (w/v) BSA binding buffer for 1 hour and then labeled with streptavidin HRP (1:10000) for another hour. ECL assay was done and signal was exposed on an X-ray film.

Results in Fig. 3.23 show that the biotinylated primer could label the total cell lysate from different cell types non-specifically in a similar way.

3.4.2 Heating effect on the non-specific binding

Binding of aptamer to its target is believed to be mediated by the three-dimensional structure of the aptamer which is sequence specific. To test if the non-specific binding was a result of the recognition of the cytosolic proteins with the sequence specific structure of the oligonucleotide, the primers were removed by heating at 95 °C for 3 minutes.

In this experiment, the Western blotted membrane was first labeled with forward primers, and then streptavidin HRP. The membrane was washed in binding buffer for three times 15 minutes each, once at room temperature, once at 95 °C and then the last one at room temperature again. After washing, streptavidin HRP was then loaded to the membrane again.

Results of the assay after heating treatment are illustrated in Fig. 3.24. It can be seen from the results that the binding between aptamers and cytosolic proteins could not be dissociated by heating. This suggests that the non-specific binding may not be mediated by sequence-dependent secondary structures of the oligonucleotides. It may be a result of charge-charge interaction between the DNA and protein or due to the presence of proteins which could bind DNA molecules on the membrane.

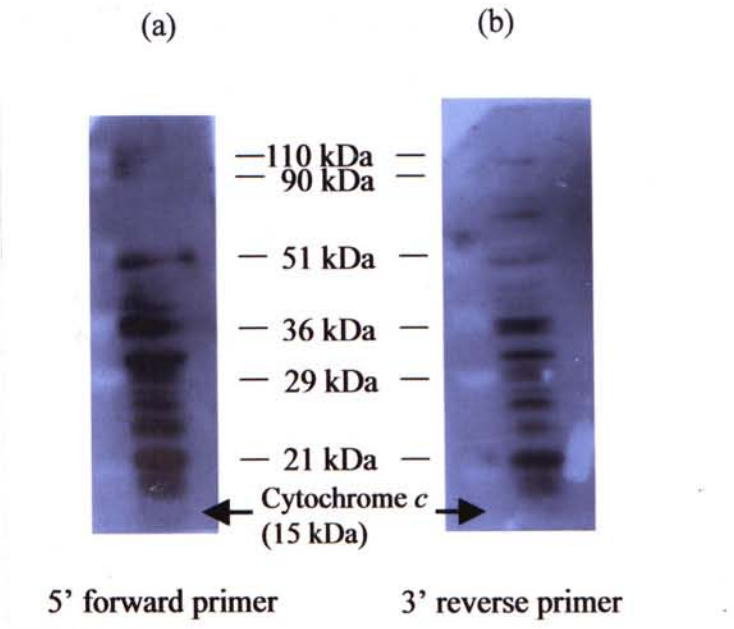


Fig. 3.24. Heating effect on the non-specific binding of cellular proteins to biotinylated 5' forward primers.

Total cell lysate of L929 was extracted with lysis buffer containing a high dose of digitonin (400 μg/ 4 x 10^6 cells). Cellular proteins were separated by 15% SDS-PAGE and transferred to PVDF membranes. Non-specific binding was blocked by using 1% (w/v) BSA in binding buffers. The membranes was then labeled with 100 pmol biotinylated 5' forward primers or 3' reverse primers as indicated in 1% (w/v) BSA binding buffers for 1 hour and then labeled with streptavidin HRP (1:10000) for another hour. After that the membrane was washed three times at room temperature, 95 °C and then room temperature again. Streptavidin HRP (1:10000) was added and ECL assay was done.

3.4.3 Using milk as a blocking agent

3.4.3.1 Milk blocked membrane

For Western blotting, BSA 1% (w/v) and milk 5% (w/v) are commonly used as blocking agents. In this part of the experiment, 5% (w/v) milk was used instead of 1% (w/v) BSA to block the PVDF membrane after the proteins were transferred to the membrane. Western blot analysis was performed as before.

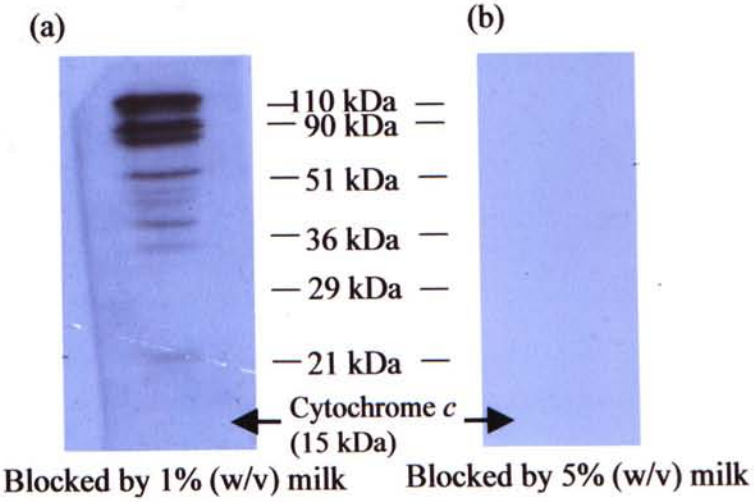


Fig. 3.25. Effect of milk as a blocking agent.
Total cell lysate of L929 was extracted with lysis buffer containing a high dose of digitonin (400 µg/ 4 x 10^6 cells). Cellular proteins were separated by 15% SDS-PAGE and transferred to PVDF membranes. Non-specific binding was blocked by using (a) 1% or (b) 5% (w/v) milk. The membranes was then labeled with 100 pmol biotinylated 5' forward primer in (a) 1 % or (b) 5% (w/v) milk binding buffer for 1 hour and then labeled with streptavidin HRP (1:10000) for another hour. ECL assay was done and signal was exposed on an X-ray film.

Results from Fig. 3.25 indicate that the binding of oligonucleotides to cellular proteins could not be blocked by 1% (w/v) milk but could be eliminated by 5% (w/v) milk effectively.

3.4.3.2 Milk prevented the binding of aptamer to cytochrome *c*

From the results above, it was found that nothing can be probed in the presence of 5% (w/v) milk. There are two possible explanations for this observation. First, milk may dissociate the non-specific binding between proteins and oligonucleotides. Alternatively, it can prevent the binding between the proteins and oligonucleotides.

To explore these possibilities, Western blot analysis with aptamer was performed as described before by using 1% (w/v) BSA as blocking agent. After the ECL assay, the membrane was then incubated with 5% (w/v) milk at 4 ^{o}C overnight. On another day, anti-streptavidin HRP was loaded again. For another membrane, the cell lysate was probed with biotinylated 5' forward primer together with 5% (w/v) milk and then with streptavidin HRP.

Results in Fig. 3.26 indicate that the binding between proteins and the oligonucleotides could not be dissociated after incubation with 5% (w/v) milk overnight. However, the 5% milk could prevent the binding between the proteins and the oligonucleotides (Fig. 3.26c).

(a) (b) (c)

Fig. 3.26. Effect of milk as a blocking agent on the aptamer-protein binding.

Total cell lysate of L929 was extracted with lysis buffer containing a high dose of digitonin (25 μg/ 4 x 10^6 cells). Cellular proteins were dotted on PVDF membranes. Non-specific binding was blocked by 1% (w/v) BSA. (a) The membranes were then labeled with biotinylated 5' forward primer in 1% (w/v) BSA in binding buffer for 1 hour and then labeled with streptavidin HRP (1:10000) for another hour. (b) The membrane from (a) was incubated in 5% (w/v) milk binding buffer overnight at 4 °C, and then labeled with streptavidin HRP (1:10000) for another hour. (c) Another membrane labeled with biotinylated 5' forward primer in 5% milk in binding buffer for 1 hour and then labeled with streptavidin HRP (1:10000) for another hour. ECL assay was done and signal was exposed on X-ray films.

3.4.3.3 Cell lysate labeled with cy-3 after SDS-PAGE by using milk as blocking agent

As 5% milk was found to block the binding between cellular proteins and 5' primers effectively, cy-3 was tried to label with cell lysate after SDS-PAGE by using 5% milk as a blocking agent.

Fig. 3.27. Binding of cy-3 with cell lysate after SDS-PAGE by using 5% milk as a blocking agent.

Total cell lysate of L929 was extracted with lysis buffer containing a high dose of digitonin (25 μg/ 4 x 10^6 cells). Cellular proteins were dotted on PVDF membranes. Non-specific binding was blocked by using 5% (w/v) milk. The membranes were then labeled with 100 pmol gel purified cy-3 in 5% (w/v) milk in binding buffer for 1 hour and then labeled with streptavidin HRP (1:10000) for another hour. ECL assay was done and signal was exposed on an X-ray film.

Results in Fig. 3.27 showed that milk blocked all the binding of cy-3 with cytosolic proteins suggesting that cy-3 could not labeled cytochrome *c* in the presence of 5% milk. It may be due to the fact that the selection of cy-3 was not performed in a condition with milk, so it could not recognize cytochrome *c* in this new condition.

3.4.3.4 Aptamer selection against cytochrome *c* in the presence of milk

In order to select and aptamer for cytochrome *c* with the use of milk as a blocking agent, the selection was started again by using 100 pmol oligonucleotides with 40 random nucleotides. In this new selection process, the pool was first counter selected with a PVDF membrane blocked by 5% milk to eliminate those oligonucleotides that could bind to the milk components. The oligonucleotides remained were transferred to and incubated with a PVDF membrane which was saturated with 20 μg/ml denatured cytochrome *c* and blocked with 5% milk. After washing with binding buffer, the bound aptamers were eluted by using 40 μg/ml native cytochrome *c*. After PCR amplification, the PCR products were separated by 12% polyacrylamide 7M urea denaturing gel (data not shown). Oligonucleotides with suitable size were found suggesting that by 5% (w/v) milk blocked cytochrome *c* on PVDF membrane could select aptamer from the pool.

In Western blot analysis, the proteins were denatured that their conformations may be different from that of native forms and they may be recognized by different aptamers. In this set of selection, the cytochrome *c* on the PVDF membrane was denatured by mixing with 2X SDS-loading buffers which contained SDS and boiling for 5 minutes before dotted on membrane. Using denatured cytochrome *c* as a target of aptamer selection, we should be able to select those oligonucleotides that could bind to the denatured cytochrome *c* in the Western blotting. Moreover, during the selection, the aptamers bound to the denatured cytochrome *c* were eluted by native cytochrome *c*. These eluted aptamers were collected and should be able to bind to both native and denatured cytochrome *c*.

After 5 rounds of selection, the aptamers selected were amplified and biotinylated. The biotinylated aptamers were used to label the cell lysate after SDS-PAGE by using 5% milk as a blocking agent.

From the results of Fig. 3.28, it was found that the aptamers selected bound cytosolic proteins but not cytochrome *c* if 1% BSA was used as blocking agent while they did not bind to cytochrome *c* and other cytosolic protein if 5% milk was used.

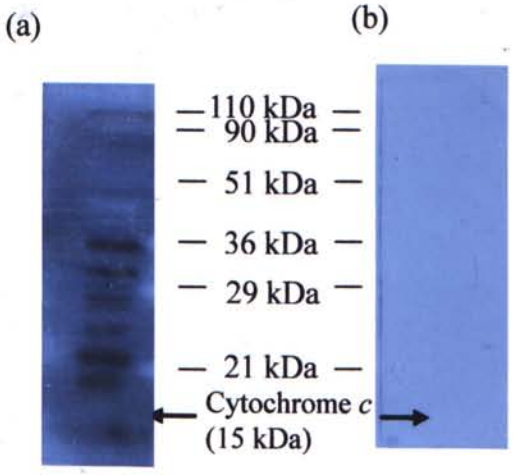


Fig. 3.28. Binding of the aptamer selected in the presence of milk with cell lysate after SDS-PAGE.

Total cell lysate of L929 was extracted with lysis buffer containing a high dose of digitonin (25 μg/ 4 x 10^6 cells). Cellular proteins were dotted on PVDF membranes. Non-specific binding was blocked by using (a) 1% BSA (w/v) or (b) 5% (w/v) milk. The membranes was then labeled with 100 pmol gel purified aptamer selected in the presence of milk in (a) 1% (w/v) BSA or (b) 5% (w/v) milk in binding buffer for 1 hour and then labeled with streptavidin HRP (1:10000) for another hour. ECL assay was done and signal was exposed on an X-ray film.

3.4.4 Using DNA as a Blocking agent

3.4.4.1 DNA blocked the non-specific binding

From the results in Fig. 3.14 and Fig. 3.24, the non-specific binding of the oligonucleotides to the cytosolic proteins seems to be sequence-independent. With the use of different oligonucleotides of different sequences, the outcome in terms of labeling pattern with the total cell lysate was similar. This may be due to the presence of proteins on the membrane which could bind DNA molecules.

To test this possibility, we tried to use calf thymus DNA to block the non-specific binding before the addition of biotinylated forward primer. As shown in Fig. 3.29, 100 μg DNA could partially block the binding of the primers to the cytosolic proteins and 150 μg could totally remove the binding.

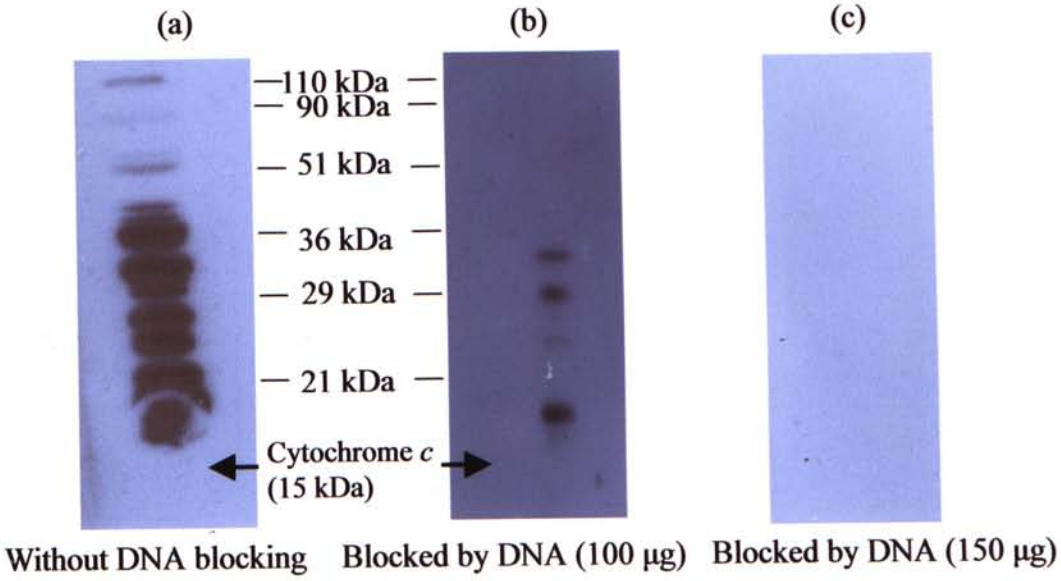


Fig. 3.29. The use of DNA as blockers to eliminate non-specific binding from biotinylated 5' forward primer.

L929 total cell lysate was extracted with lysis buffer containing a high dose of digitonin (400 µg/ 4 x 10^6 cells). Cellular proteins were then separated by 15% SDS-PAGE and transferred to PVDF membranes. Non-specific binding was blocked by using 1% (w/v) BSA. The membranes were then incubated without (a) or with (b) 100 µg or with 150 µg (c) sonicated calf thymus DNA in 1% (w/v) BSA binding buffer for 1 hour. The membranes were then labeled with biotinylated 5' forward primers also in 1% (w/v) BSA binding buffer for 1 hour and then labeled with streptavidin HRP (1:10000) for another hour. ECL assay was done and signal was exposed on X-ray films.

3.4.4.2 Cell lysate labeled with cy-3 after SDS-PAGE by using DNA as blocking agent

From the results above, DNA could block all the non-specific binding. Next, we tried to use calf thymus DNA as a blocking agent in the Western analysis with different monoclonal aptamers (cy-1 to cy-4) against the total cell lysates. Results from Fig. 3.30 indicate that aptamer cy-1 to cy-4 could not label cytochrome *c* and cytosolic proteins after blocking with DNA.

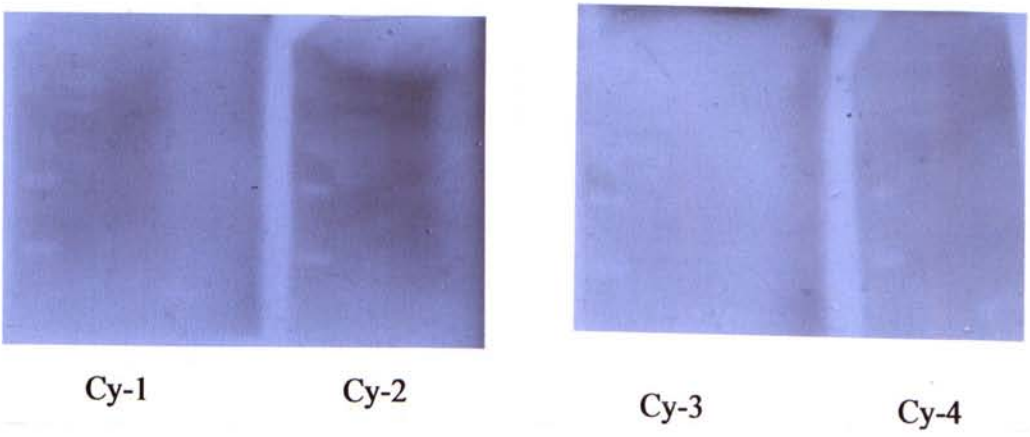


Fig. 3.30. The use of DNA as blockers to eliminate non-specific binding from cy-1 to cy-4.

Total cell lysate of L929 was extracted with lysis buffer containing a high dose of digitonin (400 μg/ 4 x 10^6 cells). Cellular proteins were then separated by 15% SDS-PAGE and transferred to PVDF membranes and non-specific binding was blocked by using 1% (w/v) BSA in binding buffers. The membranes were then blocked with 75 μg/ml calf DNA in binding buffer and then labeled with cy-1 to cy-4 as indicated in 1% (w/v) BSA binding buffers for 1 hour and then labeled with streptavidin HRP (1:10000) for another hour. ECL assay was done and signal was exposed on X-ray films.

3.4.4.3 Selection against cytochrome *c* blocked by DNA

From Fig. 3.30, aptamer cy-1 to cy-4 could not label cytochrome *c* and cytosolic proteins after blocking with DNA. This may be due to the fact that the presence of DNA on the PVDF membrane interfered the binding between aptamers cy-1 to cy-4 to the cytochrome *c*.

To improve this situation, aptamers were selected against cytochrome *c* coated on PVDF membrane with DNA as blocking agent. After selection, the aptamers obtained were amplified and biotinylated by PCR with excess biotinylated 5' forward primer. After PCR amplification, the PCR products were checked by 12% polyacrylamide 7M urea denaturing gel (data not shown). Oligonucleotides with suitable size were present showing that aptamers could be selected from the library by DNA blocked cytochrome *c* on the PVDF membrane. The biotinylated PCR product were then purified by 12% polyacrylamide 7M urea denaturing gel. The purified aptamers were tested for binding by Western analysis (Fig. 3.31).

From Fig. 3.31, it was found that the aptamers selected against cytochrome *c* blocked by DNA still could not label cytochrome *c* and other cytosolic proteins. This may due to the fact that the calf DNA used for blocking are with different sequences, they may block the binding of cytochrome *c* and cellular proteins with different sequence. Alternatively, calf thymus DNA reacted with the selected aptamer and the complexes were washed away during the Western analysis. And thus, the calf thymus DNA may interfere the recognition between the aptamers and target.

Fig. 3.31. Binding of aptamer selection against cytochrome *c* blocked by DNA with cell lysate after SDS-PAGE.

Total cell lysate of L929 was extracted with lysis buffer containing a high dose of digitonin (400 μg/ 4 x 10^6 cells). Cellular proteins were then separated by 15% SDS-PAGE and transferred to PVDF membranes and non-specific binding was blocked by using 1% (w/v) BSA in binding buffers. The membranes were then blocked with 75 μg/ml calf DNA in binding buffer and then labeled with the aptamers selected in 1% (w/v) BSA binding buffers for 1 hour and then labeled with streptavidin HRP (1:10000) for another hour. ECL assay was done and signal was exposed on an X-ray film.

3.4.4.4 Labeling of cell lysate treated with DNase, RNase or both after SDS-PAGE

Another possibility of generating non-specific bindings between our aptamers and total cell lysate is the presence of DNA and RNA which interact with the aptamers. In order to eliminate the possible bindings of aptamers to the cellular DNA and RNA in the total cell lysates, the total cell lysate was pre-treated with DNase or RNase or both to eliminate all the DNA and RNA that may be present in the system which interact with aptamers before dotted on PVDF membrane (Fig. 3.32).

From the results in Fig. 3.32, it is clear that those treated with DNase or RNase still produced signals with the primers as the one observed in control (that was not treated with DNase or RNase). These suggest that the DNA and RNA in the total cell lysate did not play an important role in generating the non-specific binding.

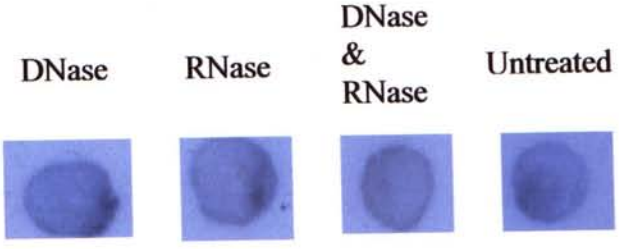


Fig. 3.32. Effect of DNase and RNase on the binding of biotinylated 5' forward primer with total cell lysate.

Total cell lysate of L929 was extracted with lysis buffer containing a high dose of digitonin (400 μg/ 4 x 10^6 cells). The cell lysate were then treated with DNase, RNase or both (20 μg/ ml each at 37 °C for 1 hour) as indicated. The treated or untreated cell lysates were dotted on the PVDF membrane. After the proteins were coated on the membrane, non-specific binding was blocked by 1% (w/v) BSA. The membranes was then labeled with 100 pmol biotinylated 5' forward primer in 1% BSA binding buffers for 1 hour and then labeled with streptavidin HRP (1:10000) for another hour. ECL assay was done and signal was exposed on an X-ray film.

3.5 Photo-SELEX

To increase the binding specificity and affinity, Photo-SELEX was used in this set of selection. In the Photo-SELEX process, thymidine in the random region was replaced by 5-halo-deoxyuridine. Oligonucleotides with 5-halo-deoxyuridine can form covalent cross linkages with specific aromatic amino acids such as Tyr or Trp of the antigen under UV light. This covalent bonding can enhance the binding specificity (Meisenheimer *et al.*, 2000).

3.5.1 Selection process

Forty pmol of the pool of photo-oligonucleotides were first incubated with cytochrome *c* (final concentration 1.5 nM) in 800 µl binding buffer at room temperature for 15 minutes with shaking. After washing, the mixture was then irradiated by UV light to form cross linkage. The cross linkage complexes were then concentrated by using filter and separated by 12% polyacrylamide 7 M urea denaturing gel. The cross linking between the photo oligonucleotide and cytochrome *c* was covalent which could not be broken down by heat or urea in the gel. The presence of this complex can be seen as a mobility shift in the electrophoresis gel when compared to the one without cross-linkages.

As a control, the photo-oligonucleotide pool was incubated with cytochrome *c* and followed the same steps of signal development but without UV light irradiation. The gel photo (Fig. 3.33) shows that after irradiation of UV light, the photo oligonucleotide formed complex with the target proteins and ran slower in the denaturing gel. However, for the control, it did not show any delay suggesting that no complex was formed when no irradiation of UV light was performed. The band

which ran slower was cut out, crashed and incubated with 400 μl proteinase K (400 μg/ml) in TE buffer at 37 °C overnight to separate the proteins from the oligonucleotides. The oligonucleotides were then extracted and amplified by PCR.

From the gel photo, there was a band with the same size of the marker. However, a smear was observed on the top of this band suggesting a mobility shift of a large complex with the aptamer. Moreover, except the high molecular weight of large complexes, there were a faster band with the same size as the pool and a smear below it. The presence of the faster band may be a result of the pool of oligonucleotides which was in excess amount to the target proteins. Some oligonucleotides remain freely in the reaction. And the present of a smear which have smaller size may due to the present of oligonucloetide fragment which broke down from the pool.

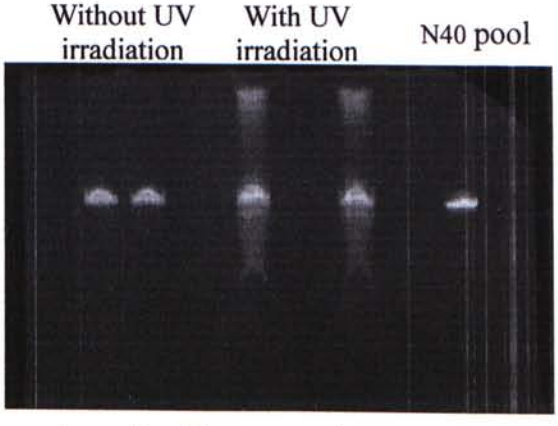


Fig. 3.33. A denaturing gel with urea to show the effect of UV light on the binding of photo-oligonucleotides to target proteins.

The photo oligonucleotides incubated with cytochrome *c* illuminated with (for selection) or without (as control) UV light for 2 minutes. After concentration, urea and 6X loading dye were added and the mixture was heated at 95 °C for 3 minutes before loading to the gel. N40 pool was run simultaneously as the size marker. After separated by 12% polyacrylamide 7M urea denaturing gel at 100V for 45 minutes, DNA was stained by ethidium bromide and visiblized by UV light.

3.5.2 Cell lysate labeled with photo-aptamer

The photo-oligonucleotides after 5 rounds of selection were used to probe with cytochrome *c* together with cellular proteins by Western blot analysis. As shown in Fig. 3.34a and b, a Western blot with 1% (w/v) BSA, they both showed the binding with cytochrome *c* and cytosolic proteins. And the one with UV irradiation (Fig. 3.34b) showed more non-specific bands than the one without (Fig. 3.34a). On the other hand, it was found that the photo-aptamers could not label cytochrome *c* and cytosolic proteins before and after UV irradiation in the presence of 5% (w/v) milk (Fig. 3.34c and d).

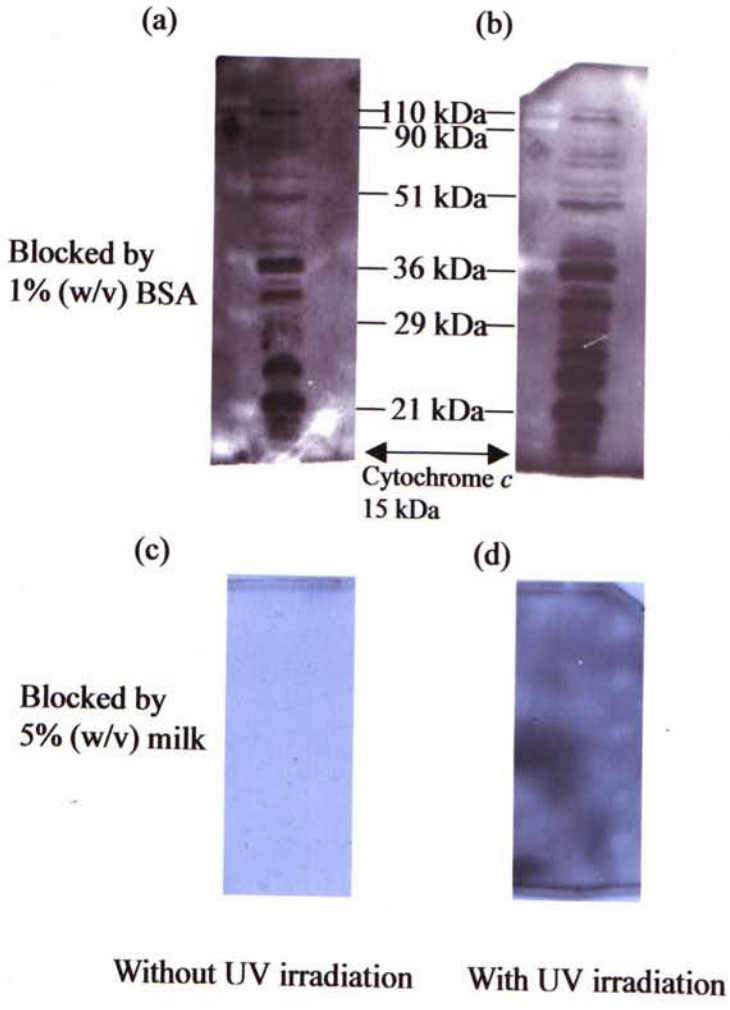
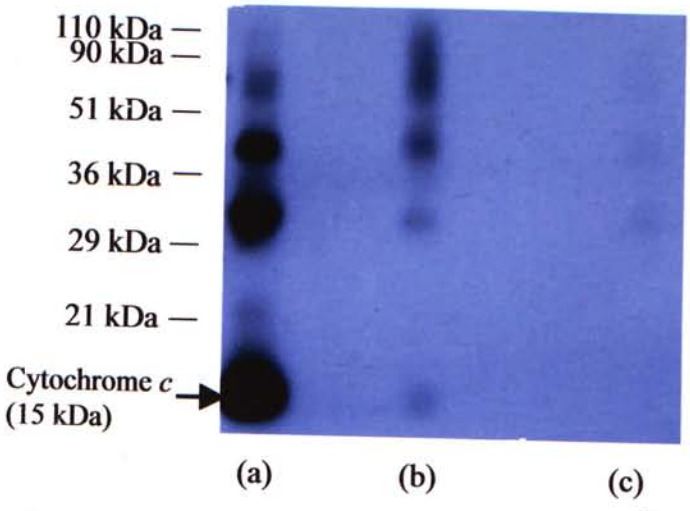


Fig. 3.34. A Western blot to show the effect of UV light on the binding of photo-oligonucleotides to target protein.

Total cell lysate of L929 was extracted with lysis buffer containing a high dose of digitonin (400 μg/ 4 x 10^6 cells). Cellular proteins were separated by 15% SDS-PAGE and transferred to PVDF membranes. Non-specific binding was blocked by using (a, b) 1% (w/v) BSA or (c, d) 5% (w/v) milk. The membranes were then labeled with 100 pmol photo-aptamer in binding buffer with blocking agent for 1 hour. For the one with UV irradiation (b,d), it was excited by UV for 2 minutes after washing the membrane once with binding buffer. The two membranes were then labeled with streptavidin HRP (1:10000) for another hour. ECL assay was done and signal was exposed on X-ray films.

3.5.3 Testing by immunoprecipitation

To test the binding of photo-aptamer to cytochrome *c*, we tried to investigate whether the photo-aptamer can absorb cytochrome *c* or not? To test this, cytochrome *c* was obtained by immunoprecipitation. After amplification and biotinylation, the photo-aptamers were conjugated on streptavidin paramagnetic beads. The beads were then incubated with native cytochrome *c* for 15 minutes and illuminated with UV light for 2 minutes in a system with a fixed amount of cytochrome *c*. After washing, the beads were then incubated with DNase at 37 °C for half an hour. The proteins were then added with equal volume of 2X SDS loading buffer and boiled for 5 minutes. After that, the proteins were analyzed by using Western blotting with anti-cytochrome *c* antibody. As shown in Fig. 3.35, photo-aptamers could pick up cytochrome *c*. However, the photo-aptamers seemed to pick up more of the high molecular weight forms than the 15 kDa of cytochrome *c* suggesting that the high molecular weight cytochrome *c* was also the target protein of the photo-aptamer with a high affinity.



(a) 1.5 μg cytochrome *c*
(b) cytochrome *c* after immunoprecipitated by photo-aptamer
(c) cytochrome *c* after immunoprecipitated by biotinylated 5' forward primer

Fig. 3.35. Immunoprecipitation by using photo-aptamer.

Cytochrome *c* (1.5 μg) were immunoprecipitated by using photo-aptamers conjugated on paramagnetic beads and illuminated with UV for 2 minutes. After incubation, the oligonucleotides were digested by DNase (10 mg/ml) to release the cytochrome *c* bound. The proteins were separated by 15% SDS-PAGE and transferred to PVDF membranes. Non-specific binding was blocked by using 5% (w/v) milk. The membranes were then labeled with anti-cytochrome *c* antibody in 5% (w/v) milk binding buffer (1:2000) for 1 hour and then labeled with HRP conjugated anti-mouse antibody (1:2000) for another hour. ECL assay was done and signal was exposed on X-ray films.

3.6 Application

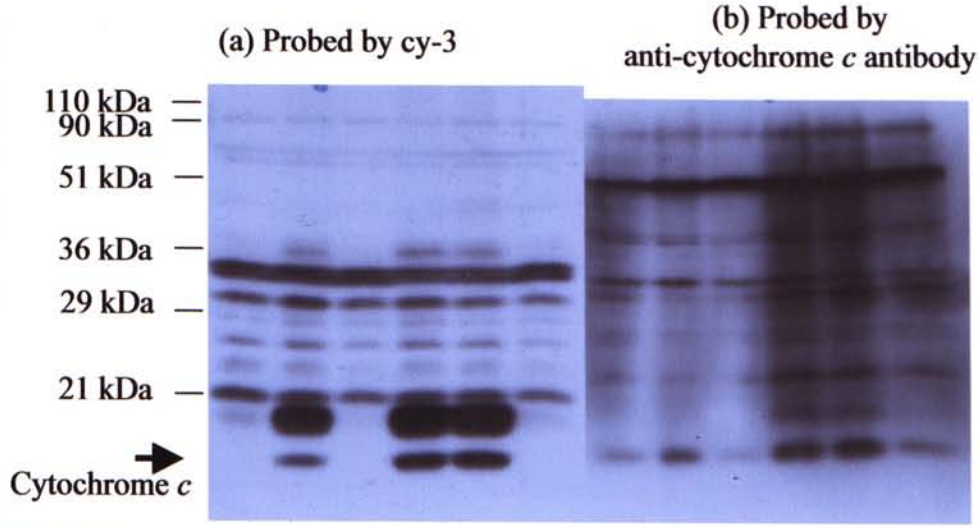
3.6.1 Detection of the cytochrome *c* in cytosolic proteins after treatment of TNF-α

Although we could not eliminate the non-specific binding between the monoclonal aptamer with cellular proteins by different experimental designs, the semi-quantitation against cytochrome *c* after SDS-PAGE can be performed if we concentrate on the cytochrome *c* bands only. To investigate this, we tried to use aptamer to probe the cytochrome *c* released from TNF-α treated L929 cells.

TNF-α induces apoptosis in L929 cells and this process involves the release of cytochrome *c* from mitochondria. The release of cytochrome *c* can be detected by anti-cytochrome *c* antibody or aptamer in the cytosol of cells. After treatment of cells with TNF-α, the cytosolic proteins of L929 cells were carefully extracted with lysis buffer containing a low dose of digitonin (12.5 μg/ 4 x 10^6 cells) which only permeabilized the plasma membrane but not the mitochondrial membrane. The proteins collected in the supernatant were then separated by SDS-PAGE. The presence of cytochrome *c* was detected by using aptamer cy-3 and anti-cytochrome *c* antibody (Fig. 3.36).

From the result in Fig. 3.36, it was found that both the aptamer and antibody could detect the release of cytochrome *c* from mitochondria in the TNF-α treated cells when compared with the control. In the untreated control, no and only a small amount of cytochrome *c* were observed when probed with antibody or aptamer respectively suggesting that both agents are specific for cytochrome *c*. Unfortunately, both of them showed non-specific binding to the cytosolic proteins other than

cytochrome *c*. However, both the aptamer and antibody produced a time-dependent signals in the cells treated with TNF-α for various time intervals. The longer the treatment with TNF-α, the more the cytochrome *c* was released into the cytosol which appeared in the supernatant after the treatment with a low dose of digitonin.



TNF (50 ng/ml)	-	+	-	+	+	-	-	+	-	+	+	-
Time of Treatment (hrs)	3	3	6	6	9	9	3	3	6	6	9	9

Fig. 3.36. Cytochrome *c* released in TNF-α treated L929 cells probed with cy-3 and anti-cytochrome *c* antibody.

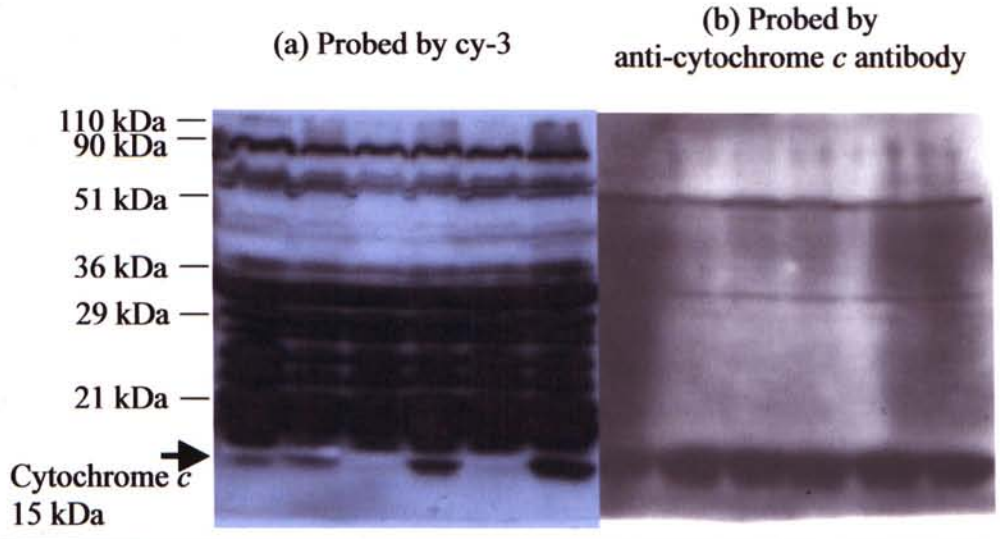
L929 were seeded in a 6 wells plate (1 x 10^6 cells per well) overnight. The cells were then incubated with medium alone (control) or TNF-α (50 ng/ml) for 3, 6 or 9 hours at 37 °C, 5% CO_2. After treatment, the cells were washed with PBS and cytosolic cell lysate was extracted with lysis buffer containing a low dose of digitonin (12.5 μg/ 4 x 10^6 cells). Cellular proteins were then separated by 15% SDS-PAGE. The separated proteins were then transferred to PVDF membranes. Non-specific binding was blocked by 1% (w/v) BSA. The membranes were then labeled with (a) cy-3 in 1% (w/v) BSA binding buffer (1:400) or (b) anti-cytochrome *c* in 1% (w/v) BSA TBS-T (1:1000) for 1 hour and then labeled with streptavidin HRP (1:10000) or anti-mouse HRP conjugated antibody (1:1000) respectively for another hour. ECL assay was done and signal was exposed on X-ray films.

3.6.2 Detection of the cytochrome *c* in total cell lysate after treatment of TNF-α

From the results above, the release of cytochrome *c* from mitochondria could be detected by the anti-cytochrome *c* antibody or aptamer from the cytosolic proteins without the permeabilization of mitochondria. In this part, total cell lysate were extracted from L929 cells after treatment of TNF-α by lysis buffer with a high dose of digitonin (400 μg/ 4 x 10^6 cells) which permeabilized the whole cell including the mitochondrial membrane. All the cytochrome *c* in the cells before or after released from the mitochondria would be collected. The proteins collected were then separated by SDS-PAGE. And the presence of cytochrome *c* was detected by using aptamer cy-3 and anti-cytochrome *c* antibody (Fig. 3.37).

In the total cell lysate, it should contain all the cytochrome *c* in the cells no matter they are present in the mitochondria or cytosol. The amount of the cytochrome *c* in the same number of cells should be the same. From the results probed with antibody (Fig. 3.37 b), it can be seen that the levels of cytochrome *c* in total cell lysate before and after treatment of TNF-α for 3, 6, and 9 hours were more or less the same. However, from the results probed with cy-3, it was found that the levels of cytochrome *c* in the total cell lysate of the TNF-α treated group was not the same as that of the untreated group (Fig. 3.37 a). The level and pattern of cytochrome *c* in Fig. 3.37a were in a similar pattern as the one observed in Fig. 3.36. This suggests that aptamer cy-3 could recognize the cytochrome *c* released from mitochondria with a high affinity than the one in the mitochondria. This discrepancy may be due to the fact that cytochrome *c* released from mitochondria has conformational change after the induction of apoptosis (Jemmerson *et al*, 1999). Therefore, it is possible that cy-3 may have a selective binding ability to the form of

cytochrome *c* that is released from the mitochondria of apoptotic cells.



TNF (50 ng/ml)	-	+	-	+	-	+	-	+	-	+	-	+
Time of Treatment (hrs)	3	3	6	6	9	9	3	3	6	6	9	9

Fig. 3.37. Cytochrome *c* in total lysate of L929 cells with or without treatment of TNF-α probed with cy-3 and anti-cytochrome *c* antibody.

L929 were seeded in a 6 wells plate (1 x 10^6 cells per well) overnight. The cells were then incubated with medium alone (control) or TNF-α (50 ng/ml) for 3, 6 or 9 hours at 37 °C, 5% CO_2. After treatment, the cells were washed with PBS and total cell lysate were extracted with lysis buffer containing a high dose of digitonin (400 μg/ 4 x 10^6 cells). Cellular proteins were then separated by 15% SDS-PAGE. The separated proteins were then transferred to PVDF membranes. Non-specific binding was blocked by 1% (w/v) BSA. The membranes were then labeled with (a) cy-3 in 1% (w/v) BSA binding buffer (1:400) or (b) anti-cytochrome *c* antibody in 1% (w/v) BSA TBS-T (1:1000) for 1 hour and then labeled with streptavidin HRP (1:10000) or anti-mouse HRP conjugated antibody (1:1000) respectively for another hour. ECL assay was done and signal was exposed on X-ray films.

3.6.3 Detection of cytochrome *c* in different cellular compartments after treatment of TNF-α

To confirm these interesting results, we tried to lyse cells with different dosages of digitonin. Under this experimental condition, the degrees of permeabilization of cell membranes are different. With lysis buffer with a low dose of digitonin, only cytosolic proteins can be obtained; while that with a high dose of digitonin, the proteins in the mitochondria can also be extracted.

After treatment of L929 cells with TNF-α or medium alone for 9 hours, proteins were extracted from the same group of cells by lysis buffer stepwisely with increasing dosages of digitonin starting from 12.5 μg of digitonin. In this preparation, an increasing amount of cytochrome *c* should be obtained in the TNF-α untreated cells with an increasing dose of digitonin. The proteins were then separated by SDS-PAGE and labeled with anti-cytochrome *c* antibody or monoclonal aptamer cy-3.

From the results labeled with anti-cytochrome *c* antibody (Fig. 3.38), it can be seen that cytochrome *c* mainly localized in the mitochondria (with the highest dose of digitonin used) of the cells without TNF-α treatment but located in the cytosol (extracted with the lowest dose of digitonin) after the treatment of TNF-α. In particular, a cytochrome *c* band with the highest intensity was observed in lane D' of Fig. 3.38b suggested that TNF-α had released cytochrome *c* from the mitochondria to the cytosol, with a higher concentration of digitonin, not too many cytochrome *c* could further be extracted (Fig. 3.38b C' to A'). In the control group, most of the cytochrome *c* was found in lane A with the highest digitonin concentration indicating

that the concentration of digitonin less than 87.5 μg/ 4 x 10^6 cells could not permeabilize the mitochondrial membrane (Fig. 3.38b, D to A). However, for the one labeled with monoclonal aptamer cy-3, only the cytochrome *c* located in the cytosol which is released from the mitochondria after treatment of TNF-α could be labeled. Cytochrome *c* released by the highest digitonin concentration (187.5 μg) could not be recognized by cy-3 (Fig. 3.38a, lane A')

However, from the results of Western blotting labeled with cy-3 in the previous chapters, the cytochrome *c* in lysate extracted with a high dose of digitonin (400 μg/ 4X10^6 cells) from untreated cell could also be labeled. This may due to the fact that some of the cytochrome *c* was also released from mitochondria and present in cytosol in untreated cells (Fig. 36b). Also, cy-3 may labeled both release and unreleased cytochorme *c* but could bind to released form with a much higher affinity.

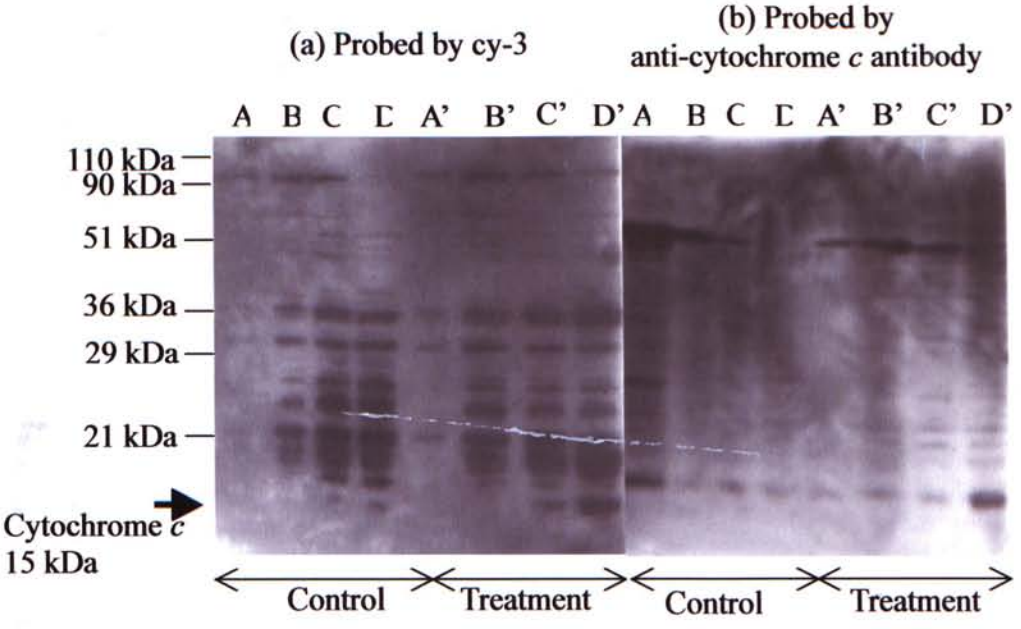


Fig. 3.38. Cytochrome *c* in cell lysate of L929 cells with or without treatment of TNF-α extracted with increasing dosage of digitonin probed with cy-3 and anti-cytochrome *c* antibody.

L929 were seeded in a 6 wells plate (1 x 10^6 cells per well) overnight. The cells were then incubated with medium alone (control) or TNF-α (50 ng/ml) for 9 hours at 37 °C, 5% CO_2. After treatment, the cells were washed with PBS and cell lysate were extracted with lysis buffer containing various amounts of digitonin starting from 12.5 μg digitonin and then stepwisely up to 187μg (A, A': 187.5, B,B':87.5, C,C': 37.5, D,D':12.5 μg digitonin/ 4 x 10^6 cells). Cellular proteins were then separated by 15% SDS-PAGE. The separated proteins were then transferred to PVDF membranes. Non-specific binding was blocked by 1% (w/v) BSA. The membranes were then labeled with (a) cy-3 in 1% (w/v) BSA binding buffer (1:400) or (b) anti-cytochrome *c* in 1% (w/v) BSA TBS-T (1:1000) for 1 hour and then labeled with streptavidin HRP (1:10000) or anti-mouse HRP conjugated antibody (1:1000) respectively for another hour. ECL assay was done and signal was exposed on X-ray films.

To find out if monoclonal aptamer cy-3 labeled the same protein as the anti-cytochrome *c* antibody, the same membrane was incubated with anti-cytochrome *c* antibody and its secondary antibody with HRP. From the ECL results, it was found that at the size of 15kDa, only one band was observed suggesting the protein band that labeled with cy-3 or antibody was the same (Fig. 3.39a).

Next, we wanted to test if cy-3 could block the binding of anti-cytochrome *c* antibody to cytochrome *c*. This was done by pre-blocked the cytochrome *c* by cy-3 before labeling the protein with the anti-cytochrome *c* antibody and its secondary antibody. In the absence of streptavidin HRP, the signal produced caused only by the antibody. From the results, it was found that not too many bands other than cytochrome *c* were obtained (Fig. 3.39a). Moreover, the cytochrome *c* band intensities (Fig. 3.39b) were more or less the same as the one labeled with antibody alone (without cy-3 pre-blocked (Fig. 3.38b)). Also, in the PVDF membrane with cy-3 and in the presence of streptavidin HRP, the Western blot was similar to Fig. 3.38a. This suggests that cy-3 could not block the binding of anti-cytochrome *c* antibody to its ligand. Taken together, our results suggest that cy-3 could differentiate the cytochrome *c* released from the mitochondria of apoptotic cells and those remained in the mitochondria as well as the apo-cytochrome *c* in the cytosol.

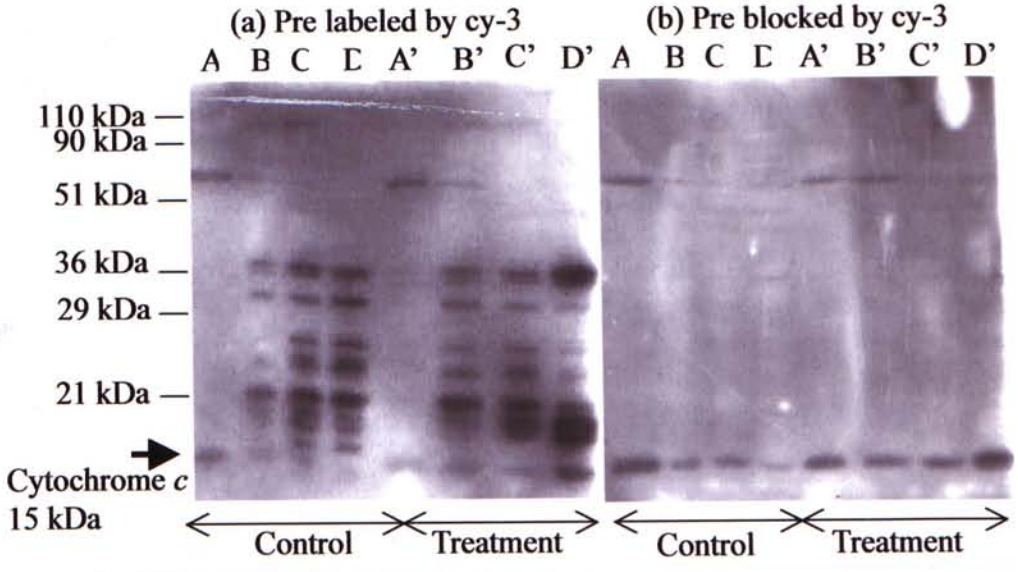


Fig. 3.39. Cytochrome *c* in cell lysate of L929 cells with or without treatment of TNF-α extracted with increasing dosage of digitonin.

L929 were seeded in a 6 wells plate (1 x 10^6 cells per well) overnight. The cells were then incubated with medium alone (control) or TNF-α (50 ng/ml) for 9 hours at 37 °C, 5% CO_2. After treatment, the cells were washed with PBS and cell lysate were extracted with lysis buffer containing various amounts of digitonin starting from 12.5 μg digitonin and then stepwisely up to 187μg (A, A': 187.5, B,B':87.5, C,C': 37.5, D,D':12.5 μg digitonin/ 4 x 10^6 cells). Cellular proteins were then separated by 15% SDS-PAGE. The separated proteins were then transferred to PVDF membranes. Non-specific binding was blocked by 1% (w/v) BSA. The membranes were then labeled with cy-3 in 1% (w/v) BSA binding buffer and then (a) with (pre-labeled) or (b) without (pre-blocked) streptavidin HRP (1:10000) for 1 hour. The two membranes were then labeled with anti-cytochrome *c* in 1% (w/v) BSA TBS-T (1:1000) for 1 hour and anti-mouse HRP conjugated antibody (1:1000) for another hour. ECL assay was done and signal was exposed on X-ray films.

Chapter 4
Discussion

4.1. General information

For more than one decade, aptamer technology has been developed and many different oligonucleotides have been selected to recognize different types of targets. These targets include drugs (Jenison RD *et al.*, 1994), amino acids (Mannironi *et al.*, 2000), proteins (Tuerk *et al.*, 1992), organic dyes, co-factors, peptides, metal ions, nucleotide base analogs and virus (Pan *et al.*, 1995) (Table 1.1). Although aptamers were selected to recognize different proteins, their specificity was not verified and they were seldom proved to bind to their target specifically from a mixture of different proteins (Radrizzani *et al.*, 1999). In my project, aptamers against cytochrome *c* were selected from a pool of oligonucleotides and they were tested to label cytochrome *c* from a mixture of proteins in a cell lysate by using Western blot analysis.

4.1.1 The pool of oligonucleotides

Starting from a DNA pool with 76 nucleotides oligonucleotides which consists of 40 random nucleotides in the central region and 18 nucleotides each at the 5' and 3' flanked ends, aptamers were selected against cytochrome *c*.

4.1.2 Design of oligonucleotides

Until now, it is still not entirely clear to know what the optimal number of nucleotides should be in the DNA library. Oligonucleotides used for the selection of aptamers usually contain 30 to 60 randomized nucleotides (Gold *et al.*, 1995).

Longer molecules seems more attractive that they provide a linear increase in the probability of finding a defined sequence and a combinatorial increase in the probability of finding two or more interacting sequences. Moreover, they will fold into more complex and stable structures than shorter ones. However, irrespective of whether shorter or longer random sequence libraries are used, the overall complexity of the population is limited by DNA synthesis chemistry to a total of 10^{13}-10^{16} different sequences. With this constrain, randomized libraries usually contain all possible sequences from 22 to 27 residues in length ($4^{22} \approx 10^{13}$, $4^{27} \approx 10^{16}$).

Under this limitation, a frequent misconception is that a longer pool will be less representative than shorter one in terms of the completeness of randomized sequences. For example, the number of possible 40-mers is $4^{40} \approx 10^{24}$ and thus a library of 10^{15} different molecules samples only 10^{-9} of 40-mer space. From this calculation, the chance of the loss of the sequences in a pool increases with the length of the sequences used. However, longer pools will subsume the shorter pools (longer pool contains all possibility of shorter pool) and in addition, they will contain a diverse and perhaps critical representation of longer sequence motifs.

In addition, in most oligonucleotide synthesis, the yield and quality of the product decrease dramatically when the total length exceeds 100 nucleotides (Shi *et al.*, 1997). After balancing the complexity and the loss of the sequence in the pool, oligonucleotides with 40 random nucleotides in between two flanked ends which

used for amplification were used in our project.

4.1.3 SELEX

SELEX composes of *in vitro* selection and amplification. *In vitro* selection has proven to be an extremely useful technique for defining protein-binding sites and for deriving novel binding species (aptamers) from random sequence populations (Famulok *et al.*, 1998, Eaton *et al.*, 1997). In our selection, the first round of selection started with 100 pmol of the pool of oligonucleotides, which contains about 6×10^{13} different sequences. Although, it could not cover all the possibility made by 40 random nucleotides, it reached the limitation of the DNA synthesis chemistry, thus, the loss of the possible sequences was reduced to minimum.

4.1.3.1 Buffer condition of selection

Buffer conditions are important to stabilize the active conformation of a target molecule. In our selection, the binding buffer we used was composed of 10 mM HEPES, 75 mM NaCl, 5 mM $MgCl_2$ and 1mM $CaCl_2$. High concentrations of monovalent cations (50 to 1000 mM are typically used) tend to quell nonspecific binding of species to targets (Binkley *et al.*, 1995). In contrary, divalent cations such as Mg^{2+} and Ca^{2+} can promote non-specific binding. However, they are important for the formation of nucleic acid secondary and tertiary structures. Thus, low concentrations of divalent ions (1 to 10 mM) are used in our project (Marshall *et al.*, 2000).

4.1.3.2 Binding equilibrium

During the selection, the binding reaction should be allowed to reach equilibrium. For almost all aptamers, this requires more seconds. However, because the association between aptamers and their targets may involve an induced fit mechanism (Westhof *et al.*, 1997), it is wise to allow the binding reaction to equilibrate from 5 to 60 minutes.

4.1.3.3 Prevalence of matrix-binding species

In our selection, aptamers were selected against cytochrome *c* coated on PVDF membrane. Some nucleic acids were found to bind to modified cellulose filters in a target-independent manner (Tuerk *et al.*, 1992). Also, if the stringency of the selection employing filter binding as a separation technique is too high at the beginning or the stringency is increased too quickly in subsequent rounds, filter-binding species always stands out. To avoid this problem, a negative, selection step (counter selection) is frequently employed.

4.2. Selection

In our selection, the non-specific site on PVDF membrane was blocked by 1% (w/v) BSA. And the oligonucleotides were first counter-selected against a BSA blocked PVDF membrane. That is, those sequences that bound to BSA or PVDF membrane were discarded. Only the oligonucleotides remained were used to be selected against the target. With counter selection, we could make sure that only sequences bound to cytochrome *c* but not BSA or PVDF membrane were chosen.

After binding, the membrane was washed with binding buffer. This could remove those unbound or loosely bound sequences. The bound oligonucleotides were then eluted by elution buffer under heat. The oligonucleotides collected were then amplified by PCR.

4.2.1 Cycle numbers of PCR

Throughout the amplification of PCR, the aptamers were amplified with the primers which were complementary to the 5' and 3' flanked ends of the oligonucleotides. To amplify the single-stranded sense oligonucleotides, excess 5' forward primer (30 times more than 3' reverse primer) was added to the PCR reaction. Theoretically, only those oligonucleotides with the same size with the aptamers could be produced. However, from the gel photo (Fig. 3.1) of PCR products produced by different PCR cycles, there were some high molecular weight products produced in the later rounds of PCR. However, the reason of the production

of these high molecular weight products is still unknown but these products did not interfere with the eventual isolation of high affinity aptamers (Cox *et al.*, 2000). To prevent these unwanted products, the PCR cycle number was optimized and our results indicated that 15 cycles was the optimal number (Fig. 3.1).

4.3. Assay of aptamers selected

4.3.1 The use of biotin-streptavidin for recognition

After seven rounds of selection, aptamers selected were amplified and biotinylated by PCR with the use of excess biotinylated 5' forward primer. By this arrangement, single-stranded sense oligonucleotides were amplified and conjugated with biotin at 5' end. The biotin could be recognized by streptavidin with high affinity (Gretch *et al.*, 1987). With suitable substrate, colour product or chemiluminescence can be produced by the HRP conjugated on the streptavidin. These signals could then be detected by plate reader, x-ray film or fluorescence plate reader.

4.3.2 Polyclonal aptamers

Using dot blot assay, cytochrome *c* dotted on the PVDF membrane was labeled with the aptamers. Our results show that the aptamers selected could label cytochrome *c* in a dose-dependent manner, and they did not bound BSA which acted as a blocking agent on the PVDF membrane (Fig. 3.2).

4.3.3 Monoclonal aptamer

After SELEX, the aptamers obtained were polyclonal. They consisted of many oligonucleotides with different sequences in the core region. In the polyclonal aptamers, it might contain some sequences that had lower affinity to the target or that could bind to the matrix. Thus, monoclonal aptamers were obtained by cloning

technique.

4.3.4 Cy-3 shows the highest affinity to cytochrome *c*

From the polyclonal aptamers, 9 monoclonal aptamers were obtained successfully (Fig. 3.3). By the binding test of cy-1 to cy-4, cy3 was found to have the highest affinity to cytochrome *c* among the four (Fig. 3.4). Cy-3 was then used for further tests. By dot blot analysis and Western blot of pure cytochrome *c*, the detection limitation of cy-3 in our experimental conditions was found to be 0.25 and 0.5 μg (Fig. 3.5 and Fig. 3.8) respectively. By the test of ELISA, cy-3 showed a linear relationship with the amount of cytochrome *c* in the range of 0-4 μg/ 100 μl per well and became level off when the amount of cytochrome *c* was greater than 4 μg (Fig. 3.6). Cy-3 was also found to bind cytochrome *c* from different species such as horse, rat, tuna, yeast and chicken (Fig. 3.9). However, cy-3 could not block the binding between cytochrome *c* and anti-cytochrome *c* antibody (Fig. 3.7). This suggests that the cy-3 and antibody have different recognition sites on the cytochrome *c* molecule.

4.3.5 The presence of non-specific binding

Next, we tested whether cy-3 was able to bind cytochrome *c* from a mixture of proteins. Using lysis buffer with a high dose of digitonin (400 μg/ 4 x 10^6 cells), all the proteins in the cells including cytochrome *c* in the mitochondria could be extracted. From our results, it was found that cy-3 could bind to cytochrome *c* and

cytosolic proteins from a mixture of proteins (Fig. 3.10). This may be due to the fact that cy-3 could bind to some of the cytosolic proteins non-specifically. However, after testing the other 8 monoclonal aptamers, the results showed that all of them bound to cytochrome *c* and other cytosolic proteins with the same pattern (Fig. 3.11).

4.4. Counter selection against cell lysate

In the antibody production, all the B clones are screened before maturation and the B clones and their antibodies that recognize self proteins are deleted from our immune system. This clonal deletion can prevent auto-immunity in our body. However, oligonucleotides used for selection contained all the random sequences which might contain some aptamers that could recognize the cellular proteins that are not immunogenic.

To overcome this problem, oligonucleotides were counter selected against cell lysate to exclude those oligonucleotides which could recognize cellular proteins from the pool. Proteins for counter selection were extracted from cells with a low dose of digitonin (12.5 μg /4 x 10^6 cells). Using this low dose of digitonin, only the proteins in the cytosol but not organelles (such as cytochrome *c* in mitochondria) were extracted. Therefore, oligonucleotides bound to cytosolic proteins but not cytochrome *c* were removed by the counter selection.

In my project, three different methods were examined to remove those oligonucleotides that could bind cytosolic proteins. The first one is the counter selection against cytosolic proteins as mentioned before. Before the selection, oligonucleotides were first incubated with a membrane coated with cytosolic proteins (extracted with a low dose of digitonin) followed by a second selection against cytochrome *c*.

The second one is by the use of fixed cells. After cells were fixed by 0.3% glutaraldehyde in PBS, the membranes were permeabilized and oligonucleotides could freely enter the cells. After incubation, some of the oligonucleotides would bind to their specific targets including cytochrome *c* in the cells. After washing away those unbound oligonucleotides, cytochrome *c* was added to elute the aptamers that bound to cytochrome *c* specifically. During the elution, cytochrome *c* was added and incubated with the fixed cells with decreasing time intervals to increase the stringency of selection.

The stringency of selection was affected by numerous variables. Shortening the incubation time interval is one of the methods. In the early round of selection, less stringent conditions and longer incubation periods were used to maximize the recovery of the relatively few functional molecules present in the initial pool. Stringency was then increased in the later rounds as the population winnowed to predominantly functional molecules.

From our results, both the aptamers selected with counter selection against cytosolic proteins and selected by fixed cells were found to bind to cytosolic proteins and cytochrome *c* (Fig. 3.12 and Fig. 3.13). This suggests that counter selection was not clean enough. Thus, we tried the third method. Theoretically, those aptamers bind to the cytochrome *c* on the PVDF membrane after PAGE should be the oligonucleotides that bound to cytochrome *c* specifically. Thus, the aptamers were

eluted from the band of cytochrome *c* after the Western blot analysis. The aptamers eluted were then reamplified and tested for the binding with the cell lysate again. Unfortunately, the aptamers eluted from the bands of cytochrome *c* still showed non-specific binding to cytosolic proteins (Fig. 3.14).

4.5. Primer testing

From the results in Figs. 3.10 - 3.14, they all showed non-specific binding with a similar pattern. This may be due to the fact that there might be a common item present in the labeling solution that could bind cytosolic proteins. In all the PCR reactions, biotinylated 5' forward primer was in excess and they contained the site that could be recognized by streptavidin HRP for signal generation. Thus, we next tested whether the 5' forward primer interacted with the proteins in the cell lysate.

It is clear from our result that the primer could bind to cytosolic proteins but not cytochrome *c* (Fig. 3.15). However, after we eliminated the excess primers from the PCR product before labeling, cy-3 still showed the non-specific binding to cytosolic proteins (Fig. 3.16).

To eliminate the primer sequence at the ends of aptamers, aptamers with core sequence were prepared by DNA synthesis chemistry as the aptamers could no longer be amplified from plasmids without the flanked primer ends through PCR. After sequencing, the sequences of cy-1 to cy-3 were obtained.

With the information from sequencing, the core sequence of cy-3 without flanked primer ends was synthesized with a biotin conjugated at the 5' end. After testing its binding to cell lysate, it was found that the core sequence also bound cytochrome *c* and cytosolic proteins (Fig. 3.17).

In the literature, Bianchini's group (2001) was the only group that reported the development of monoclonal aptamers against cellular proteins and showed single band in Western blot analysis (Bianchini *et al.*, 2001; Radrizzani *et al.*, 1999). This suggests that their primer set might be a good starting material for aptamer development. With this thought in our mind, same primers with biotin conjugated were synthesized and their binding to cell lysate was examined.

After testing the binding of the primers of ERK2-FOR and ERK2-REV to cell lysate in the conditions we used, our results (Fig. 3.22) showed that the primers from Bianchini group also bound cytosolic proteins with the same binding pattern as we saw before. However, with the labeling conditions used by Bianchini *et al.*, (2001) (blocked the PVDF membrane with 5% (w/v) milk), his primer pair did not show any non-specific binding to the cytosolic proteins. These results suggested that the problem of the non-specific binding may be due to the inefficiency of blocking when 1% BSA (w/v) was used as blocking agent.

4.6. Sequences and secondary structures of monoclonal aptamer

According to the sequencing results, all three aptamers cy-1 to cy-3 were found to be CG rich sequences in the middle core.

The binding of aptamer to its target was known to be achieved by its three dimensional structures. However, little is known about the tertiary structures of the aptamers. As for the secondary structures, a wide variety of secondary structures were shown to be used by aptamers, for examples, stem-loops, stem-bulge-stems, pseudoknots, and G-quartets (Lorsch *et al.*, 1996).

The secondary structures of cy-1 to cy-3 with or without the flanked primer sequences were predicted by using Vienna RNA Secondary Structure Prediction and Comparison Programme (Fig. 3.18). Our results showed that the structures of the three aptamers were different but cy-2 and cy-3 were quite similar. Moreover, the secondary structures of the core sequence of cy-1 to cy-3 were quite similar corresponding to that of their whole sequences. At the moment, we do not know why these aptamers, though with different nucleotide sequence, bound cytosolic protein in a similar pattern.

From the sequencing information, we tried to introduce mutation to the aptamer cy-3. With mutation, the predicated secondary structures were found to be different

from that of cy-3 (Fig. 3.20). Theoretically, the change of the primary sequence of aptamer could affect the binding affinity of the structures. From our results (Fig. 3.21), it was found that the affinity of one of the mutants (cy-3-2) became lowered after mutation, while the other one increased (in terms of intensity). However, the binding of the aptamer to its target is mediated by the tertiary structure, the secondary structure might not show the real picture.

Most aptamers were found to have conformational change upon ligand binding. For the ATP aptamer, the cyanocobalamin aptamer (Wilson *et al.*, 1995) and the guanosine/arginine joint aptamer (Connell *et al.*, 1993), their bases become hypersensitive to chemical modification in the presence of ligand. These suggest that the aptamers undergo structural changes when they bind to their ligands. Moreover, aptamers against theophylline show major changes by NMR spectra when theophylline is present. This observation led Jenison *et al.*, (1994) to conclude that aptamer changes their structure upon ligand binding (Davies *et al.*, 1993). It was suggested that these sorts of structural changes were a common feature in RNA recognition (Lorsch *et al.*, 1996). In this connection, we do not know whether our aptamers showed similar property.

4.7. Elimination of non-specific binding

4.7.1 Non-specific binding may be mediated by sequence-independent recognition

The folding of oligonucleotide can be destroyed by heat at 95 °C, and it will become linear in structure. From the results of heat dissociation, the non-specific binding could not be removed by heating at 95 °C (Fig. 3.24). This indicated that the non-specific binding might not be mediated by the sequence-dependent recognition of the oligonucleotides. It may be a result of charge-charge interactions between the DNA and proteins or due to the presence of proteins which could bind DNA molecules on the PVDF membrane.

4.7.2 Elimination of non-specific binding by milk

Next, we tested the use of 1% milk and 5% milk as blocking agents. From our results, 5% milk could block the non-specific binding of the 5' forward primers effectively (Fig. 3.25). However, 5% milk could also block the binding of cy-3 to cytochrome *c* (Fig. 3.27).

The folding of oligonucleotide depends on the condition of buffer such as pH and salt concentration. Lorsch *et al.*, (1994) showed that the aptamers against cyanocobalamin could only form complexes in the buffer conditions in which they were originally selected; alteration in the buffer conditions for selection led to an alteration of aptamer sequences. This means that the conditions under which the

selection is carried out will largely determine what binding species are isolated.

When non-fat milk was used as blocking agents, the composition of the binding buffer such as calcium, magnesium, zinc and phosphorous was changed which affects the conformations of oligonucleotides.

From our results, it was found that milk could not dissociate the non-specific binding of the primers to the cytosolic proteins but it prevented the binding between them (Fig. 3.26). After the salt concentration of binding buffer had been changed, the conformations of the oligonucleotides should be altered. As our results showed that milk could not dissociate the non-specific binding, this further suggested that the non-specific binding was not mediated by the sequence-specific recognition.

As mentioned before, the condition of binding buffer may alter the conformation of the oligonucleotides and different oligonucleotides may be selected to the same target in different buffer conditions. Thus, the monoclonal aptamer selected with BSA might not be suitable for the use in the presence of milk. Therefore, we started another set of selection with the same condition of labeling in the presence of milk. In this set of selection, oligonucleotides after counter selection against milk were allowed to bind to the denatured cytochrome *c* which was in the same form as in Western blotting (e.g. with SDS after boiling). Subsequently, the bound oligonucleotides were eluted with the use of native cytochrome *c*. With this

strategy, the aptamers selected were supposed to bind to both the native and denatured cytochrome *c*. However, from the results of Western blotting, nothing could be labeled with the aptamers selected (Fig. 3.28).

4.7.3 Eliminate the non-specific binding by using DNA

As indicated by the results of dissociation of non-specific binding by milk and heating, the binding between proteins and oligonucleotides may be mediated by the sequence-independent binding. In this part, DNA was used as blocking agent to reduce the binding of oligonucleotides to DNA-binding proteins in the cell lysate. From our results, it was found that 150 μg DNA could block the non-specific binding effectively (Fig. 3.29). However, similar to the results with milk, DNA could block the binding of cy-1 to cy-4 to cytochrome *c* (Fig. 3.30).

The presence of DNA used as blocking agent might interfere the recognition site of aptamers cy-1 to cy-4 to the cytochrome *c*. In the new set of selection, the target was blocked with 1% BSA and then with DNA before selection. From our results, it was found that nothing could be labeled with the aptamers again (Fig. 3.31).

There was one possibility that nucleic acid might be present in the cell lysate during protein extraction that interacts with our aptamers. To remove the nucleic acid in the cell lysate, DNase, RNase or both were used to treat the lysate before testing

for the non-specific binding. From our results, it was found that the nucleic acid in the cell lysate did not play an important role in the non-specific binding (Fig. 3.32).

4.8. Photo-aptamer

Photo-aptamers were shown to have high selectivity and specificity. By replacing thymidine with 5'-bromo-2'-deoxyuridine (Meisenheimer *et al.*, 1997), photo-aptamer can form photo-cross linking with the aromatic and sulfur-bearing amino acid residues of the protein targets which are in suitable proximity (Ito *et al.*, 1980; Swanson *et al.*, 1981; Dietz *et al.*, 1987). The photo-aptamers against recombinant human basic fibroblast growth factor$_{155}$ (bFGF$_{155}$) have been successfully selected (Golden *et al.*, 1999). The bFGF$_{155}$ specific photo-aptamers have a high sensitivity for bFGF$_{155}$ when compared with the commercially available monoclonal antibodies (Golden *et al.*, 2000).

In this set of selection, a fixed ratio of oligonucleotides to proteins was allowed to react. If a large amount of target is available for binding, then more aptamers with lower association constants will be retained. Thus, the amount of oligonucleotides should be in excess when compared to that of the targets. In our experiment, 40 pmol oligonucleotides were incubated with 1.2 pmol cytochrome *c* in each round of selection, and their ratio was about 33:1.

After five rounds of selection, photo-aptamers were selected against cytochrome *c*. From the results, it was found that the photo-aptamers could bind to cytochrome *c* with 1% BSA (w/v) with or without UV irradiation. However, for the one labeled in the presence of 5% milk, the non-specific binding was blocked and

photo-aptamers could not label the cytochrome *c* in the presence or absence of UV irradiation (Fig. 3.34). It seems likely that photo-aptamer could not be used to solve the problem of non-specific binding. After adding the photo-aptamers, oligonucleotides that loosely bound on the PVDF membrane were not washed away and formed cross linking with the protein under UV irradiation (Fig. 3.34).

Another test was employed to test the photo-aptamers. From the results of immuno-precipitation, it showed that photo-aptamer could pick up cytochrome *c* especially its polymeric form while the 5' biotinylated forward primer could not (Fig. 3.35).

4.9. Application of the monoclonal aptamer cy-3

After all these methods, we still could not eliminate the non-specific binding between the monoclonal aptamer with cellular proteins. Next, we tried to concentrate ourselves on the cytochrome *c* band for the semi-quantification of cytochrome *c* with SDS-PAGE. To investigate this, we tried to use aptamer cy-3 again to probe the cytochrome *c* released from TNF-α treated L929 cells.

Cytochrome *c* is a water soluble protein locates in the space between the inner and outer mitochondrial membrane. The precursor of cytochrome *c*, apocytochrome *c*, is synthesized in the cytoplasm. Upon translocation into the mitochondria, cytochrome *c* refolds and acquires a heme moiety required for functionality in the mitochondrial respiration chain. The heme-bound form of cytochrome *c* is called holocytochrome *c*. Cytochrome *c* is an essential component of the mitochondrial respiratory chain (Liu *et al.*, 1996) and it also plays an important role in apoptosis. Apoptotic stimulus triggers the release of cytochrome *c* from the mitochondria into cytosol where it binds to Apaf-1. The cytochrome *c*/Apaf-1 complex activates caspase-9, which then activates caspase-3 and other downstream caspases.

4.9.1 Aptamer can label cytochrome *c* as antibody does

TNF-α was found to induce the release of cytochrome *c* from the mitochondria by using anti-cytochrome *c* antibody (Yuen *et al.*, 2000). From our results, it was found that although the monoclonal aptamer cy-3 had non-specific binding to other

cytosolic proteins, it could label cytochrome *c* as antibody did.

To further confirm the target of cy-3 is cytochrome *c*, the cy-3 labeled protein band was cut out. We tried to sequence the N-terminus of the polypeptide chain in the 15 kDa band by Edman reaction. In Edman reaction, the polypeptide chain reacts with phenylisothiocyanate to form a covalent bond to the NH_2-terminal amino acid. Under acidic condition, this derivative catalyzes an intramolecular cyclization that results in a cleavage of the NH_2-terminal amino acid from the polypeptide chain as a phenylthiophydantoin derivative. This NH_2-terminal amino acid derivative can be separated chromatographically and identified against standards. The polypeptide chain minus the NH_2-terminal amino acid is then isolated, and the Edman reaction is repeated to identify the next NH_2-terminal amino acid. Unfortunately, our sequencing failed to produce any signal. It is well received that if a polypeptide chain was modified such as the addition of saccharide, the presence of the saccharide may block the reaction with phenylisothiocyanate and disrupt the sequencing of the protein. In fact, cytochrome *c* was found to have an acetylation at its N-terminus that affected the peptide sequencing failed (Margoliash E *et al.*, 1961).

To show the release of cytochrome *c* from mitochondria to the cytosol, the plasma membrane was carefully permeabilized by a low dose of digitonin (12.5 μg/ 4×10^6 cells). Cy-3 was able to demonstrate the release of cytochrome *c* from the mitochondria of cells treated with TNF-α in a time-dependent manner (Fig. 3.34).

In mitochondria, cytochrome *c* was believed to have interaction with the phospholipid-rich inner mitochondrial membrane (Salamon *et al.*, 1996). When cytochrome *c* associates with synthetic anionic phospholipids vesicles, it shows conformational change. This involves an opening of the heme crevice resulting from the loss of both the iron-methionine 80 ligation (Spooner *et al.*, 1992) and H-bonds between amino acid side chains and one of the heme propionates (Soussi *et al.*, 1990), as well as the destabilization of the tertiary structure of the polypeptide detected by the accessibility of the backbone amides to hydrogen-deuterium exchange (Muga *et al.*, 1991). By a monoclonal antibody 1D3 which recognizes the omega loop region around residue 44 located to the right and below the exposed heme crevice, a conformational change in cytochrome *c* was observed in apoptotic or necrotic cells but not in healthy cells (Jemmerson *et al.*, 1999). This conformationally altered cytochrome *c* appears to be important in the apoptotic cell death.

When we tried to use the monoclonal aptamer cy-3 to label the total lysate of cells treated with TNF-α, to our surprise, the results showed that cy-3 could label the cytochrome *c* in the total cell lysate (Fig. 3.37a) in a pattern similar to the one found in the cytosolic proteins (Fig. 3.36a). In contrast, anti-cytochrome *c* antibody showed a constant intensity in the cytochrome *c* bands from the cells with different treatments (Fig. 3.37b). This suggests that cy-3 could differentiate the cytochrome *c* released from mitochondria with those remained in the organelles. Mechanistically,

aptamer cy-3 might have a higher affinity to the cytochrome *c* with the conformational change after the induction of apoptosis (Fig. 3.37).

Suen *et al.*, (2000) had showed that lysis buffer with different dosages of digitonin could isolate the cellular proteins in different compartments of cells. The higher the dosage of digitonin, the higher the degree of permeabilization of the cell membrane and the cell proteins from the inner organelles of the cells can be extracted. From the results with anti-cytochrome *c* antibody, it was found that cytochrome *c* was mainly localized in the organelles presumably the mitochondria that require a high dose of digitonin to permeabilize in the cells without TNF-α treatment. For the cells with TNF-α treatment, cytochrome *c* was found in the cytosol and could be extracted with a lower dose of digitonin (Fig. 3.38 b). However, results from the monoclonal aptamer cy-3 indicated that only the cytochrome *c* located in the cytosol released from the mitochondria was labeled (Fig. 3.38 a).

To confirm this observation, cytosolic proteins from apoptotic L929 cells were probed with cy-3 and subsequently with anti-cytochrome *c* antibody. The anti-cytochrome *c* we used is from clone 7H8.2C12, its epitope is between amino acids 93-104 of pigeon cytochrome *c* and it can recognize cytochrome *c* from horse, human, mouse, pigeon and rat. Our results showed that the cytochrome *c* band originally not be seen in the lanes of untreated group at 15 kDa appeared when anti-cytochrome *c* antibody was used (Fig. 3.39a). This suggests that cytochrome *c*

existed in the gel when probed with aptamer cy-3. Moreover, the prior binding of cy-3 to cytochrome *c* did not affect the binding of antibody (Fig. 3.39b) indicating that the recognition site of cytochrome *c* by antibody and that of aptamers were different. In fact, this had been shown in the competitive ELISA assay (Fig. 3.6). Taken together, cy-3 could differentiate the intact cytochrome *c* and those released from apoptotic cells.

4.10. Conclusion I

Monoclonal aptamers were selected against cytochrome *c*. However, they showed some non-specific bindings to other cytosolic proteins possibly through a sequence-independent manner. Although these non-specific bindings could be blocked by 5% (w/v) milk or DNA, aptamer was failed to generate signals against its ligand cytochrome *c* under these condition.

In out study, it was found that our cy-3 bound to cytochrome *c* at a different site from that by anti-cytochrome *c* antibody (clone no. 7H8.2C12). By focusing on the band of cytochrome *c*, monoclonal aptamer could still be used for semi- quantization by Western blot analysis. Cy-3 was found to recognize the cytochrome *c* after apoptosis induction with a high specificity. This provides a unique property to our cy-3 for the analysis of cytochrome *c* release during apoptosis.

4.11. Conclusion II

Started from the early 1990s, aptamer technology was developed and many different aptamers have been selected for a wide range of targets. Automated selection process was also developed in 2000 (Cox *et al.*, 2001). However, it seems likely that not all the molecules can be used as the target for selection. Stanlis *et al.*, (2003) has reported that they failed to identify aptamer against EGFP according to the procedure of SELEX and even with the help of a company which specialize in the aptamer selection. The reason for this was unknown.

Actually, the technology of aptamer was still at its infant stage. For example, we do not know the three-dimensional folding mechanism of the oligonucleotide, the relationship between the binding and the structure of aptamer, how the buffer conditions affect the folding and the binding of the aptamer etc. These questions are waiting to be solved. From the literatures of aptamer selection, there are many different ideas and conditions for the selection process and thus, the process of selection is still need to be optimized.

Among the aptamers selected, some were proved to have the ability to distinguish compounds with similar structures. However, they were seldom proved to pick up their targets from a mixture of proteins (Bianchini *et al.*, 2001). This observation suggests that aptamer will play a complementary role but not replace the existing immunodiagnostic methods at this moment (Rye *et al.*, 2000). For this

young technology, more research work is needed to make it perfect. Our aptamers for cytochrome *c* are good examples to demonstrate this point.

Chapter 5
References

Backer, M. V. & Backer, J. M. (2001) 'Functionally active VEGF fusion proteins'. *Protein Expr.Purif.* 23(1):1-7.

Ball, H., Sandrasagra, A., Tang, L., Van Scott, M., Wild, J. & Nyce, J. (2003) 'Clinical Potential of Respirable Antisense Oligonucleotides (RASONs) in Asthma'. *Am.J.Pharmacogenomics.* 3(2):97-106.

Baron, V., De Gregorio, G., Krones-Herzig, A., Virolle, T., Calogero, A., Urcis, R. & Mercola, D. (2003) 'Inhibition of Egr-1 expression reverses transformation of prostate cancer cells in vitro and in vivo'. *Oncogene* 22(27):4194-4204.

Bianchini, M., Radrizzani, M., Brocardo, M. G., Reyes, G. B., Gonzalez, S. C. & Santa-Coloma, T. A. (2001) 'Specific oligobodies against ERK-2 that recognize both the native and the denatured state of the protein'. *J.Immunol.Methods* 252(1-2):191-197.

Binkley, J., Allen, P., Brown, D. M., Green, L., Tuerk, C. & Gold, L. (1995) 'RNA ligands to human nerve growth factor'. *Nucleic Acids Res.* 23(16):3198-3205.

Borgstrom, P., Bourdon, M. A., Hillan, K. J., Sriramarao, P. & Ferrara, N. (1998) 'Neutralizing anti-vascular endothelial growth factor antibody completely inhibits angiogenesis and growth of human prostate carcinoma micro tumors in vivo'. *Prostate* 35(1):1-10.

Bridonneau, P., Chang, Y. F., Buvoli, A. V., O'Connell, D. & Parma, D. (1999) 'Site-directed selection of oligonucleotide antagonists by competitive elution'. *Antisense Nucleic Acid Drug Dev.* 9(1):1-11.

Bridonneau, P., Chang, Y. F., O'Connell, D., Gill, S. C., Snyder, D. W., Johnson, L., Goodson, T., Jr., Herron, D. K. & Parma, D. H. (1998) 'High-affinity aptamers selectively inhibit human nonpancreatic secretory phospholipase A2 (hnps-PLA2)'. *J.Med.Chem.* 41(6):778-786.

Brody, E. N., Willis, M. C., Smith, J. D., Jayasena, S., Zichi, D. & Gold, L. (1999) 'The use of aptamers in large arrays for molecular diagnostics'. *Mol.Diagn.* 4(4):381-388.

Brummelkamp, T. R., Bernards, R. & Agami, R. (2002) 'A system for stable expression of short interfering RNAs in mammalian cells'. *Science* 296(5567):550-553.

Bruno, J. G. & Kiel, J. L. (2002) 'Use of magnetic beads in selection and detection of biotoxin aptamers by electrochemiluminescence and enzymatic methods'. *Biotechniques* 32(1):178-3.

Burgstaller, P., Girod, A. & Blind, M. (2002) 'Aptamers as tools for target prioritization and lead identification'. *Drug Discov.Today* 7(24):1221-1228.

Burke, D. H. & Gold, L. (1997) 'RNA aptamers to the adenosine moiety of S-adenosyl methionine: structural inferences from variations on a theme and the reproducibility of SELEX'. *Nucleic Acids Res.* 25(10):2020-2024.

Capaccioli, S., Quattrone, A., Schiavone, N., Calastretti, A., Copreni, E., Bevilacqua, A., Canti, G., Gong, L., Morelli, S. & Nicolin, A. (1996) 'A bcl-2/IgH antisense transcript deregulates bcl-2 gene expression in human follicular lymphoma t(14;18) cell lines'. *Oncogene* 13(1):105-115.

Chen, C. H., Chernis, G. A., Hoang, V. Q. & Landgraf, R. (2003) 'Inhibition of heregulin signaling by an aptamer that preferentially binds to the oligomeric form of human epidermal growth factor receptor-3 '. *Proc.Natl.Acad.Sci.U.S.A.*

Connell, G. J., Illangesekare, M. & Yarus, M. (1993) 'Three small ribooligonucleotides with specific arginine sites'. *Biochemistry* 32(21):5497-5502.

Cox, J. C. & Ellington, A. D. (2001) 'Automated selection of anti-protein aptamers'. *Bioorg.Med.Chem.* 9(10):2525-2531.

Davies, J., Ahsen U.V. & Schroeder, R. (1993) 'Antibiotics and the RNAs world: a role for low-molecular-weight effectors in biochemical evolution', In: The RNA World, R.F. Gesteland and J.F. Atkins, Editor. (Cold Spring Harbor: Gold Spring Harbor Laboratory Press) pp. 185-204.

Davis, K. A., Abrams, B., Lin, Y. & Jayasena, S. D. (1996) 'Use of a high affinity DNA ligand in flow cytometry'. *Nucleic Acids Res.* 24(4):702-706.

Deng, Q., German, I., Buchanan, D. & Kennedy, R. T. (2001) 'Retention and separation of adenosine and analogues by affinity chromatography with an aptamer stationary phase'. *Anal.Chem.* 73(22):5415-5421.

Dietz, T. M. & Koch, T. H. (1987) 'Photochemical coupling of 5-bromouracil to tryptophan, tyrosine and histidine, peptide-like derivatives in aqueous fluid solution'. *Photochem.Photobiol.* 46(6):971-978.

Drolet, D. W., Moon-McDermott, L. & Romig, T. S. (1996) 'An enzyme-linked oligonucleotide assay'. *Nat.Biotechnol* 14(8):1021-1025.

Eaton, B. E. (1997) 'The joys of in vitro selection: chemically dressing oligonucleotides to satiate protein targets '. *Curr.Opin.Chem.Biol.* 1(1):10-16.

Ellington, A. D. & Szostak, J. W. (1990) 'In vitro selection of RNA molecules that bind specific ligands'. *Nature* 346(6287):818-822.

Ellington, A. D. & Szostak, J. W. (1992) 'Selection in vitro of single-stranded DNA molecules that fold into specific ligand-binding structures'. *Nature* 355(6363):850-852.

Famulok, M. & Jenne, A. (1998) 'Oligonucleotide libraries--variatio delectat'. *Curr.Opin.Chem.Biol.* 2(3):320-327.

Famulok, M., Mayer, G. & Blind, M. (2000) 'Nucleic acid aptamers-from selection in vitro to applications in vivo'. *Acc.Chem.Res.* 33(9):591-599.

Floege, J., Ostendorf, T., Janssen, U., Burg, M., Radeke, H. H., Vargeese, C., Gill, S. C., Green, L. S. & Janjic, N. (1999) 'Novel approach to specific growth factor inhibition in vivo: antagonism of platelet-derived growth factor in glomerulonephritis by aptamers'. *Am.J.Pathol.* 154(1):169-179.

Freund, F., Boulme, F., Michel, J., Ventura, M., Moreau, S. & Litvak, S. (2001) 'Inhibition of HIV-1 replication in vitro and in human infected cells by modified antisense oligonucleotides targeting the tRNALys3/RNA initiation complex'. *Antisense Nucleic Acid Drug Dev.* 11 (5):301-315.

Geiger, A., Burgstaller, P., von der, E. H., Roeder, A. & Famulok, M. (1996) 'RNA aptamers that bind L-arginine with sub-micromolar dissociation constants and high enantioselectivity'. *Nucleic Acids Res.* 24(6):1029-1036.

Gerber, H. P., McMurtrey, A., Kowalski, J., Yan, M., Keyt, B. A., Dixit, V. & Ferrara, N. (1998) 'Vascular endothelial growth factor regulates endothelial cell survival through the phosphatidylinositol 3'-kinase/Akt signal transduction

pathway. Requirement for Flk-1/KDR activation'. *J.Biol.Chem.* 273(46):30336-30343.

Gold, L. (1995) 'Oligonucleotides as research, diagnostic, and therapeutic agents'. *J.Biol.Chem.* 270(23):13581-13584.

Gold, L., Polisky, B., Uhlenbeck, O. & Yarus, M. (1995) 'Diversity of oligonucleotide functions'. *Annu.Rev.Biochem* 64:763-797.

Golden, M. C., Collins, B. D., Willis, M. C. & Koch, T. H. (2000) 'Diagnostic potential of PhotoSELEX-evolved ssDNA aptamers'. *J.Biotechnol* 81(2-3):167-178.

Golden, M. C., Resing, K. A., Collins, B. D., Willis, M. C. & Koch, T. H. (1999) 'Mass spectral characterization of a protein-nucleic acid photocrosslink'. *Protein Sci.* 8(12):2806-2812.

Green, D. R. & Reed, J. C. (1998) 'Mitochondria and apoptosis'. *Science* 281(5381):1309-1312.

Gretch, D. R., Suter, M. & Stinski, M. F. (1987) 'The use of biotinylated monoclonal antibodies and streptavidin affinity chromatography to isolate herpesvirus hydrophobic proteins or glycoproteins'. *Anal.Biochem* 163(1):270-277.

Grillone, L. R. & Lanz, R. (2001) 'Fomivirsen'. *Drugs Today (Barc.)* 37(4):245-255.

Grzmil, M., Thelen, P., Hemmerlein, B., Schweyer, S., Voigt, S., Mury, D. & Burfeind, P. (2003) 'Bax inhibitor-1 is overexpressed in prostate cancer and its specific down-regulation by RNA interference leads to cell death in human prostate carcinoma cells'. *Am.J.Pathol.* 163(2):543-552.

Haller, A. A. & Sarnow, P. (1997) 'In vitro selection of a 7-methyl-guanosine binding RNA that inhibits translation of capped mRNA molecules'. *Proc.Natl.Acad.Sci.U.S.A* 94(16):8521-8526.

Hayward, R. L., Macpherson, J. S., Cummings, J., Monia, B. P., Smyth, J. F. & Jodrell, D. I. (2003) 'Antisense Bcl-xl down-regulation switches the response to topoisomerase I inhibition from senescence to apoptosis in colorectal cancer cells, enhancing global cytotoxicity'. *Clin.Cancer Res.* 9(7):2856-2865.

Hendeles, L. & Weinberger, M. (1983) 'Theophylline. A "state of the art" review'. *Pharmacotherapy* 3(1):2-44.

Herschlag, D. & Cech, T. R. (1990) 'Catalysis of RNA cleavage by the Tetrahymena thermophila ribozyme. 1. Kinetic description of the reaction of an RNA substrate complementary to the active site'. *Biochemistry* 29(44):10159-10171.

Herschlag, D. & Cech, T. R. (1990) 'Catalysis of RNA cleavage by the Tetrahymena thermophila ribozyme. 2. Kinetic description of the reaction of an RNA substrate that forms a mismatch at the active site'. *Biochemistry* 29(44):10172-10180.

Hicke, B. J. & Stephens, A. W. (2000) 'Escort aptamers: a delivery service for diagnosis and therapy'. *J.Clin.Invest* 106(8):923-928.

Hofmann, H. P., Limmer, S., Hornung, V. & Sprinzl, M. (1997) 'Ni2+-binding RNA motifs with an asymmetric purine-rich internal loop and a G-A base pair'. *RNA* 3 (11):1289-1300.

Huang, J., Moore, J., Soffer, S., Kim, E., Rowe, D., Manley, C. A., O'Toole, K., Middlesworth, W., Stolar, C., Yamashiro, D. & Kandel, J. (2001) 'Highly specific antiangiogenic therapy is effective in suppressing growth of experimental Wilms tumors'. *J.Pediatr.Surg.* 36(2):357-361.

Hwang, B. & Lee, S. W. (2002) 'Improvement of RNA aptamer activity against myasthenic autoantibodies by extended sequence selection'. *Biochem Biophys.Res.Commun.* 290(2):656-662.

Innis, M. A., Myambo, K. B., Gelfand, D. H. & Brow, M. A. (1988) 'DNA sequencing with Thermus aquaticus DNA polymerase and direct sequencing of polymerase chain reaction-amplified DNA'. *Proc.Natl.Acad.Sci.U.S.A* 85(24):9436-9440.

Ishizaki, J., Nevins, J. R. & Sullenger, B. A. (1996) 'Inhibition of cell proliferation by an RNA ligand that selectively blocks E2F function'. *Nat.Med.* 2(12):1386-1389.

Ito, S. Saito, I. Matsuura, T. (1980) 'Acetone-sensitized photocoupling of 5-bromouridine to tryptophan derivatives via electron-transfer process' *J. Am. Chem. Soc.*102(25): 7535-7541.

Jayasena, S. D. (1999) 'Aptamers: an emerging class of molecules that rival antibodies in diagnostics'. *Clin.Chem.* 45(9):1628-1650.

Jemmerson, R., Liu, J., Hausauer, D., Lam, K. P., Mondino, A. & Nelson, R. D. (1999) 'A conformational change in cytochrome c of apoptotic and necrotic cells is detected by monoclonal antibody binding and mimicked by association of the native antigen with synthetic phospholipid vesicles'. *Biochemistry* 38(12):3599-3609.

Jenison, R. D., Gill, S. C., Pardi, A. & Polisky, B. (1994) 'High-resolution molecular discrimination by RNA'. *Science* 263(5152):1425-1429.

Kiga, D., Futamura, Y., Sakamoto, K. & Yokoyama, S. (1998) 'An RNA aptamer to the xanthine/guanine base with a distinctive mode of purine recognition'. *Nucleic Acids Res.* 26(7):1755-1760.

Kim, S. J., Kim, M. Y., Lee, J. H., You, J. C. & Jeong, S. (2002) 'Selection and stabilization of the RNA aptamers against the human immunodeficiency virus type-1 nucleocapsid protein'. *Biochem Biophys.Res.Commun.* 291(4):925-931.

Kodadek, T. (2002) 'Development of protein-detecting microarrays and related devices'. *Trends Biochem Sci.* 27(6):295-300.

Kraus, E., James, W. & Barclay, A. N. (1998) 'Cutting edge: novel RNA ligands able to bind CD4 antigen and inhibit CD4+ T lymphocyte function'. *J.Immunol.* 160(11):5209-5212.

Leamon, C. P., Cooper, S. R. & Hardee, G. E. (2003) 'Folate-liposome-mediated antisense oligodeoxynucleotide targeting to cancer cells: evaluation in vitro and in vivo'. *Bioconjug.Chem.* 14(4):738-747.

Leung, D. W., Cachianes, G., Kuang, W. J., Goeddel, D. V. & Ferrara, N. (1989) 'Vascular endothelial growth factor is a secreted angiogenic mitogen'. *Science* 246(4935):1306-1309.

Liu, X., Kim, C. N., Yang, J., Jemmerson, R. & Wang, X. (1996) 'Induction of apoptotic program in cell-free extracts: requirement for dATP and cytochrome c'. *Cell* 86(1):147-157.

Lochrie, M. A., Waugh, S., Pratt, D. G., Jr., Clever, J., Parslow, T. G. & Polisky, B. (1997) 'In vitro selection of RNAs that bind to the human immunodeficiency virus type-1 gag polyprotein'. *Nucleic Acids Res.* 25(14):2902-2910.

Lorsch, J. R. & Szostak, J. W. (1994) 'In vitro selection of RNA aptamers specific for cyanocobalamin'. *Biochemistry* 33(4):973-982.

Lorsch, J. R. & Szostak, J. W. (1996) 'In vitro selection of Nucleic acid sequences that bind small molecues' In: Combinatorial libraries synthesis, screening & application potential, Cortese R. Editor. (Walter de Gruyter, Berlin, New York) pp. 69-86.

Majerfeld, I. & Yarus, M. (1998) 'Isoleucine:RNA sites with associated coding sequences'. *RNA* 4(4):471-478.

Mannironi, C., Di Nardo, A., Fruscoloni, P. & Tocchini-Valentini, G. P. (1997) 'In vitro selection of dopamine RNA ligands'. *Biochemistry* 36(32):9726-9734.

Mannironi, C., Scerch, C., Fruscoloni, P. & Tocchini-Valentini, G. P. (2000) 'Molecular recognition of amino acids by RNA aptamers: the evolution into an L-tyrosine binder of a dopamine-binding RNA motif'. *RNA* 6(4):520-527.

Margoliash, E., Smith, E.L., Kreil, G. & Tuppy, H. (1961) 'The complete amino-acid sequence'. *Nature* 192:1125-1127.

Marshall, K. A. & Ellington, A. D. (2000) 'In vitro selection of RNA aptamers'. *Methods Enzymol.* 318(193-214.

Meisenheimer, K. M. & Koch, T. H. (1997) 'Photocross-linking of nucleic acids to associated proteins'. *Crit Rev.Biochem Mol.Biol.* 32(2):101-140.

Mignotte, B. & Vayssiere, J. L. (1998) 'Mitochondria and apoptosis'. *Eur.J.Biochem* 252(1):1-15.

Muga, A., Mantsch, H. H. & Surewicz, W. K. (1991) 'Membrane binding induces destabilization of cytochrome c structure'. *Biochemistry* 30(29):7219-7224.

Nishikawa, F., Kakiuchi, N., Funaji, K., Fukuda, K., Sekiya, S. & Nishikawa, S. (2003) 'Inhibition of HCV NS3 protease by RNA aptamers in cells'. *Nucleic Acids Res.* 31(7):1935-1943.

Nolte, A., Klussmann, S., Bald, R., Erdmann, V. A. & Furste, J. P. (1996) 'Mirror-design of L-oligonucleotide ligands binding to L-arginine'. *Nat.Biotechnol* 14(9):1116-1119.

Ostendorf, T., Kunter, U., Eitner, F., Loos, A., Regele, H., Kerjaschki, D., Henninger, D. D., Janjic, N. & Floege, J. (1999) 'VEGF(165) mediates glomerular endothelial repair'. *J.Clin.Invest* 104(7):913-923.

Pagratis, N. C., Bell, C., Chang, Y. F., Jennings, S., Fitzwater, T., Jellinek, D. & Dang, C. (1997) 'Potent 2'-amino-, and 2'-fluoro-2'-deoxyribonucleotide RNA inhibitors of keratinocyte growth factor'. *Nat.Biotechnol* 15(1):68-73.

Pan, W., Craven, R. C., Qiu, Q., Wilson, C. B., Wills, J. W., Golovine, S. & Wang, J. F. (1995) 'Isolation of virus-neutralizing RNAs from a large pool of random sequences'. *Proc.Natl.Acad.Sci.U.S.A* 92(25):11509-11513.

Pavski, V. & Chris, L., X (2003) 'Ultrasensitive protein-DNA binding assays'. *Curr.Opin.Biotechnol* 14(1):65-73.

Petach, H. & Gold, L. (2002) 'Dimensionality is the issue: use of photoaptamers in protein microarrays'. *Curr.Opin.Biotechnol* 13(4):309-314.

Potyrailo, R. A. & Hieftje, G. M. (1998) 'Optical time-of-flight chemical detection: absorption-modulated fluorescence for spatially resolved analyte mapping in a bidirectional distributed fiber-optic sensor'. *Anal.Chem.* 70(16):3407-3412.

Radrizzani, M., Broccardo, M., Gonzalez, S. C., Bianchini, M., Reyes, G. B., Cafferata, E. G. & Santa-Coloma, T. A. (1999) 'Oligobodies: bench made synthetic antibodies'. *Medicina (B Aires)* 59(6):753-758.

Rhodes, A., Deakin, A., Spaull, J., Coomber, B., Aitken, A., Life, P. & Rees, S. (2000) 'The generation and characterization of antagonist RNA aptamers to human oncostatin M'. *J.Biol.Chem.* 275(37):28555-28561.

Rowe, D. H., Huang, J., Kayton, M. L., Thompson, R., Troxel, A., O'Toole, K. M., Yamashiro, D., Stolar, C. J. & Kandel, J. J. (2000) 'Anti-VEGF antibody suppresses primary tumor growth and metastasis in an experimental model of Wilms' tumor'. *J.Pediatr.Surg.* 35(1):30-32.

Ruckman, J., Green, L. S., Beeson, J., Waugh, S., Gillette, W. L., Henninger, D. D., Claesson-Welsh, L. & Janjic, N. (1998) '2'-Fluoropyrimidine RNA-based aptamers to the 165-amino acid form of vascular endothelial growth factor

(VEGF165). Inhibition of receptor binding and VEGF-induced vascular permeability through interactions requiring the exon 7-encoded domain'. *J.Biol.Chem.* 273(32):20556-20567.

Rusconi, C. P., Scardino, E., Layzer, J., Pitoc, G. A., Ortel, T. L., Monroe, D. & Sullenger, B. A. (2002) 'RNA aptamers as reversible antagonists of coagulation factor IXa'. *Nature* 419(6902):90-94.

Rusconi, C. P., Yeh, A., Lyerly, H. K., Lawson, J. H. & Sullenger, B. A. (2000) 'Blocking the initiation of coagulation by RNA aptamers to factor VIIa'. *Thromb Haemost* 84(5):841-848.

Rye, P.D. & Nustad, K. (2001) 'Immunomagnetic DNA aptamer assay', *Biotechniques* 30(2):290-295.

Salamon, Z. & Tollin, G. (1996) 'Surface plasmon resonance studies of complex formation between cytochrome c and bovine cytochrome c oxidase incorporated into a supported planar lipid bilayer. II. Binding of cytochrome c to oxidase-containing cardiolipin/phosphatidylcholine membranes'. *Biophys.J.* 71(2):858-867.

Seiwert, S. D., Stines, N. T., Aigner, S., Ahn, N. G. & Uhlenbeck, O. C. (2000) 'RNA aptamers as pathway-specific MAP kinase inhibitors'. *Chem.Biol.* 7(11):833-843.

Shi, H., Hoffman, B. E. & Lis, J. T. (1997) 'A specific RNA hairpin loop structure binds the RNA recognition motifs of the Drosophila SR protein B52'. *Mol.Cell Biol.* 17(5):2649-2657.

Single, B., Leist, M. & Nicotera, P. (1998) 'Simultaneous release of adenylate kinase and cytochrome c in cell death'. *Cell Death.Differ.* 5(12):1001-1003.

Skulachev, V. P. (1998) 'Cytochrome c in the apoptotic and antioxidant cascades'. *FEBS Lett.* 423(3):275-280.

Smith, D., Collins, B. D., Heil, J. & Koch, T. H. (2003) 'Sensitivity and specificity of photoaptamer probes'. *Mol.Cell Proteomics.* 2(1):11-18.

Soussi, B., Bylund-Fellenius, A. C., Schersten, T. & Angstrom, J. (1990) '1H-n.m.r. evaluation of the ferricytochrome c-cardiolipin interaction. Effect of superoxide radicals'. *Biochem J.* 265(1):227-232.

Spooner, P. J. & Watts, A. (1992) 'Cytochrome c interactions with cardiolipin in bilayers: a multinuclear magic-angle spinning NMR study'. *Biochemistry* 31(41):10129-10138.

Stanlis, K. K. & McIntosh, J. R. (2003) 'Single-strand DNA aptamers as probes for protein localization in cells'. *J.Histochem.Cytochem.* 51(6):797-808.

Suen, Y.K., Fung, K.P., Choy, Y.M., Lee, C.Y. Chan, C.W. & Kong, S.K. (2000) 'Concanavalin A induced apoptosis in murine macrophage PU5-1.8 cells through clustering of mitochondria and release of cytochrome c' *Apoptosis* 5(4): 369-377.

Swanson, B. J., Kutzer,J. C. & Koch T. H. (1981) 'Photoreduction of 5-bromouracil. Ionic and free-radical pathways'. *J. Am. Chem. Soc* 103(5): 1274-1276.

Tuerk, C. & Gold, L. (1990) 'Systematic evolution of ligands by exponential enrichment: RNA ligands to bacteriophage T4 DNA polymerase'. *Science* 249(4968):505-510.

Tuerk, C., MacDougal, S. & Gold, L. (1992) 'RNA pseudoknots that inhibit human immunodeficiency virus type 1 reverse transcriptase'. *Proc. Natl. Acad. Sci. U.S.A* 89(15):6988-6992.

Uphoff, K. W., Bell, S. D. & Ellington, A. D. (1996) 'In vitro selection of aptamers: the dearth of pure reason'. *Curr.Opin.Struct.Biol.* 6(3):281-288.

Vander Heiden, M. G. & Thompson, C. B. (1999) 'Bcl-2 proteins: regulators of apoptosis or of mitochondrial homeostasis?'. *Nat.Cell Biol.* 1(8):E209-E216.

Wallace, S. T. & Schroeder, R. (1998) 'In vitro selection and characterization of streptomycin-binding RNAs: recognition discrimination between antibiotics'. *RNA* 4(1):112-123.

Walter, G., Bussow, K., Lueking, A. & Glokler, J. (2002) ' High-throughput protein arrays: prospects for molecular diagnostics'. *Trends Mol.Med.* 8(6):250-253.

Wang, Y., Killian, J., Hamasaki, K. & Rando, R. R. (1996) 'RNA molecules that specifically and stoichiometrically bind aminoglycoside antibiotics with high affinities'. *Biochemistry* 35(38):12338-12346.

Westhof, E. & Patel, D. J. (1997) 'Nucleic acids. From self-assembly to induced-fit recognition'. *Curr.Opin.Struct.Biol.* 7 (3):305-309.

Wiegand, T. W., Williams, P. B., Dreskin, S. C., Jouvin, M. H., Kinet, J. P. & Tasset, D. (1996) 'High-affinity oligonucleotide ligands to human IgE inhibit binding to Fc epsilon receptor I'. *J.Immunol.* 157(1):221-230.

Williams, K. P., Liu, X. H., Schumacher, T. N., Lin, H. Y., Ausiello, D. A., Kim, P. S. & Bartel, D. P. (1997) 'Bioactive and nuclease-resistant L-DNA ligand of vasopressin'. *Proc.Natl.Acad.Sci.U.S.A* 94(21):11285-11290.

Wilson, C., Nix, J. & Szostak, J. (1998) 'Functional requirements for specific ligand recognition by a biotin-binding RNA pseudoknot'. *Biochemistry* 37(41):14410-14419.

Wilson, C. & Szostak, J. W. (1995) 'In vitro evolution of a self-alkylating ribozyme'. *Nature* 374(6525):777-782.

Yang, X., Bassett, S. E., Li, X., Luxon, B. A., Herzog, N. K., Shope, R. E., Aronson, J., Prow, T. W., Leary, J. F., Kirby, R., Ellington, A. D. & Gorenstein, D. G. (2002) 'Construction and selection of bead-bound combinatorial oligonucleoside phosphorothioate and phosphorodithioate aptamer libraries designed for rapid PCR-based sequencing'. *Nucleic Acids Res.* 30(23):e132.

Yuen, W. F., Fung, K. P., Lee, C. Y., Choy, Y. M., Kong, S. K., Ko, S. & Kwok, T. T. (2000) 'Hyperthermia and tumour necrosis factor-alpha induced apoptosis via mitochondrial damage'. *Life Sci.* 67(6):725-732.

Zamecnik, P. C., Goodchild, J., Taguchi, Y. & Sarin, P. S. (1986) 'Inhibition of replication and expression of human T-cell lymphotropic virus type III in cultured cells by exogenous synthetic oligonucleotides complementary to viral RNA'. *Proc.Natl.Acad.Sci.U.S.A* 83(12):4143-4146.

Zamore, P.D., Tuschl, T., Sharp, P.A. & Bartel D.P. (2000) 'RNAi: double-strand RNA directs the ATP-dependent cleavage of mRNA at 21-23 nucleotide intervals'. *Cell* 101(1): 25-33.

Zhang, X., Li, J., Li, N., Shi, X. & Li, Y. (2003) 'Safety of enteral rehabilitative therapy in rat small bowel transplantation'. *Chin Med.J. (Engl.)* 116(5):703-707.